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Technical Manual for Analysis of Organic Materials in Process Streams

Battelle Columbus Labs, Ohio

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Environmental Protection Technology Series

TECHNICAL MANUAL FOR ANALYSIS OF ORGANIC MATERIALS IN PROCESS STREAMS

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March 1976

TECHNICAL MANUAL FOR ANALYSIS OF ORGANIC MATERIALS IN PROCESS STREAMS

by

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by

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LEVEL 1 - SURVEY METHODS

INTRODUCTION

One of the major problems associated with the analysis of potentially hazardous organic emissions is the very large number of organic species which may be present in an emission sample. Additionally, the task of deciding what priority should be given to which emission source is by no means a trivial question. If comprehensive analytical methods were applied to every emission source which was suspected of being hazardous, costs would become wholly unreasonable and much effort would be wasted.

However, it is important to ensure that important emission problems do not go undetected. A very simple yet informative analytical strategy designed to address this problem is presented in this document. This strategy will provide a cost-effective survey technique which can reliably characterize emission sources, and provide input into effluent prioritization strategies.

In order that large numbers of Level-1 analytical surveys can be carried out, possibly at the same time, the procedures chosen are

deliberately simple, and can be carried out by technical staff with limited previous experience in the field. Despite their simplicity, the procedures are nevertheless highly effective at indicating whether a problem area exists.

The objective of the Level-1 analytical strategy is to provide a semi-quantitative estimation of the predominant classes of organic compounds present in a Level-1 sample. To achieve this result, the extracted sample will be subjected to liquid chromatography using step-wise solvent gradient elution in order to obtain separation of the sample into eight fractions containing the different organic classes which are present. Each fraction will subsequently be subject to gravimetric analysis in order to estimate the weight of material present. In addition, an infrared analysis of each fraction will be performed. Infrared analysis will enable the major classes of organic compounds in each of the eight fractions to be identified. Thus the ultimate result of the Level-1 analysis will be a qualitative and semi-quantitative analysis of the industrial emission sample.

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16. ABSTRACT The manual presents a very simple, yet informative, analytical strategy for the purpose of initial survey, to ensure that important emission problems do not go undetected. It also presents a more complex and detailed scheme for use on samples given high priority by the initial survey analysis. The manual was developed because of a major problem associated with the analysis of potentially hazardous organic emissions: a very large number of organic compounds may be present in a given industrial sample. If exhaustive analytical methods were applied to every emission source to be assessed, costs would become completely unreasonable, and much effort would be misdirected on samples of little concern. A comparison volume, concerned with sampling of organic substances, is available, and has been fully coordinated with the technical manual to ensure compatibility.

PRICES SUBJECT TO CHANGE

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group	
Air Pollution	Industrial	Air Pollution Control	13B	
Chemical Analysis	Processes	Stationary Sources	07D	13H
Analyzing	Hazardous Ma-	Analytical Strategy	14B	
Sampling	terials	Process Streams		13L
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Organic Compounds			07C	

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SAMPLE EXTRACTION AND RECOVERY

While it is presently anticipated that Level-1 sample acquisition will involve the same basic sampling train as Level-2 sampling, the following extraction procedures may be applied to any chosen organic sampling system. Thus there is no reason why the sample extraction procedures should not be basically similar.

The extracts of the filter, cyclones, or impactors, and the porous polymer adsorbent trap may be combined or analyzed separately after extraction. The combined or separate extracts should be reduced to a volume of about 0.5 ml by rotary evaporation.

Filter Extraction

Extraction of filter material should be carried out with methylene chloride, which is likely to extract most organic particulate matter. Extraction may be carried out using a Soxhlet apparatus for 24 hours, or by ultrasonic agitation of the filter with solvent in a sealed glass container for 1 hour, followed by conventional filtration of the shredded filter and insoluble particulate matter.

Cyclone, Impactors, and Probe Rinse

Particulate material which has been removed from a cyclone, impactor, or rinsed from a probe should be solvent extracted in a similar manner to a filter. Sohxlet extraction may be carried out in a cellulose thimble plugged with glass wool, or in a fritted (fine) glass thimble with

a glass wool plug. Ultrasonic extraction of particulate may be carried out by agitation with solvent in a sealed glass container followed by conventional filtration to remove the insoluble material.

Porous Polymer Adsorbent Trap Extraction

Porous polymer adsorbtion traps for organic species are coming into widespread use as a replacement for impinger collection because of their higher efficiency. A typical adsorbent trap is shown in Figure 1, which schematically depicts the Battelle Adsorbent Sampler. A larger version of this system will be used for sampling at flow rates up to 5 cfm or more.

Solvent extraction of chromatographic traps should be carried out by continuous solvent extraction of the adsorbent for a period of 24 hours. Such an extraction apparatus is shown in Figure 2, which depicts the continuous extraction assembly developed for the Battelle Adsorbent Sampler; the condenser and solvent reservoir are not shown. It is recommended that a similar apparatus be used for the extraction of all chromatographic adsorbent traps.

The choice of solvent for extracting a chromatographic adsorbent trap depends partly upon the nature of the adsorbent. It is important that the solvent chosen does not affect the adsorbent in any way, yet is still an effective solvent for the removal of collected material. For chromatographic adsorbent traps utilizing Tenax, extracting with pentane is recommended. The disadvantage of using a relatively inefficient solvent such as pentane is readily overcome by using the continuous extraction mode, when even polar compounds are readily extracted by the solvent.

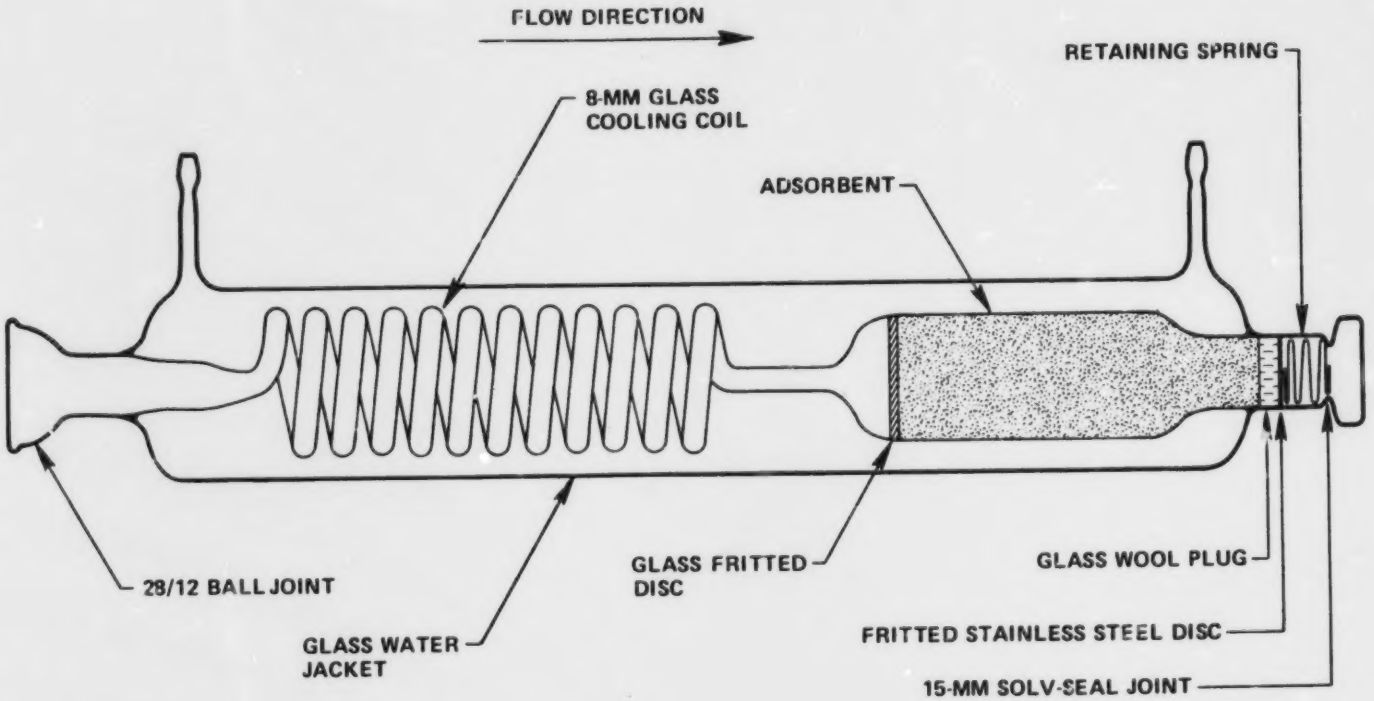


FIGURE 1. ADSORBENT SAMPLING SYSTEM

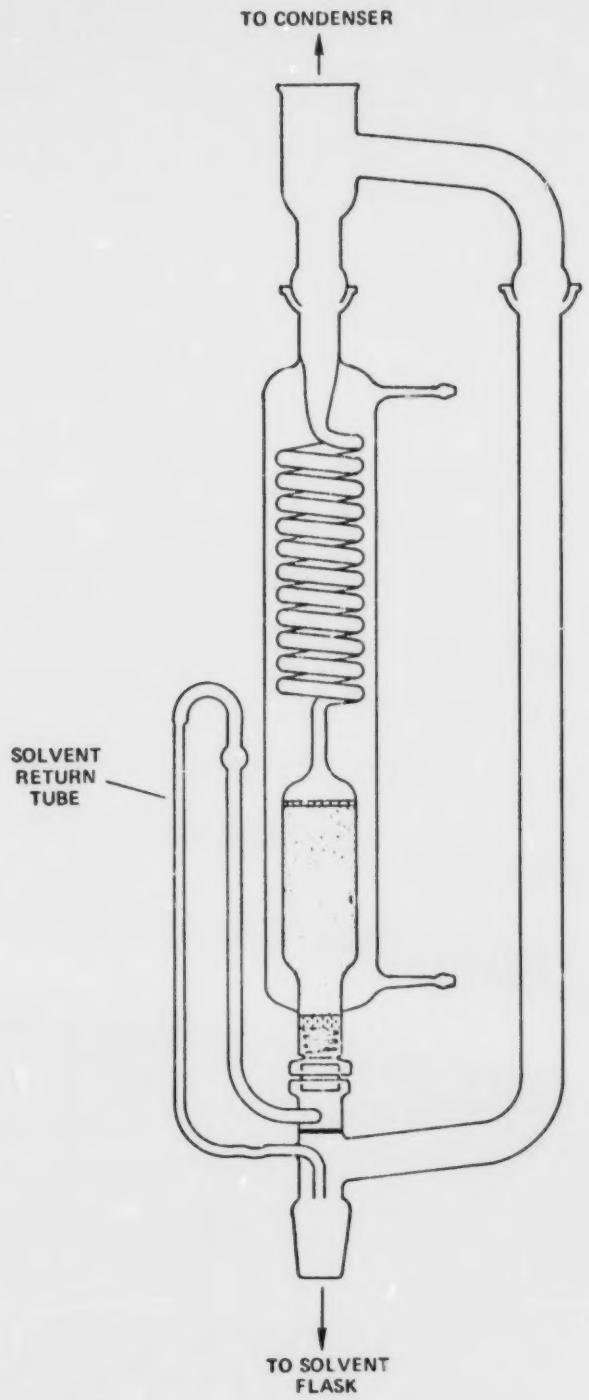


FIGURE 2. CONTINUOUS EXTRACTION ASSEMBLY FOR ADSORBENT SAMPLER

SAMPLE SEPARATION BY LIQUID CHROMATOGRAPHY

Each sample extract should be separated into eight fractions by liquid chromatography on a silica gel column. The extracts will consist of separate or combined extracts from the probe, cyclone, filter, and porous polymer trap. A supply of activated silica gel (> 200 mesh) should be prepared by freshly heating in an oven at 200 C for 24 hours.

Standard 1 x 25 cm liquid chromatography columns should be prepared as needed by partially filling the column with methylene chloride and slowly adding silica gel to a height of 23.5 cm through a funnel while agitating the column with an electric vibrator. As the column fills, sufficient methylene chloride should be added to keep the liquid level above the silica gel. When the column is filled, agitation should be continued for at least ten minutes in order to remove any air bubbles. The column should now be prepared for analytical separation by eluting the following solvents (distilled in glass) through it:

(1) 100 ml methyl alcohol

(2) 25 ml methylene chloride

(3) 25 ml 60/80 petroleum ether

The level of solvent remaining in the column should be 0.5 cm above the top of the silica gel after each solvent elution.

Sample separation into eight fractions is achieved by carefully transferring the 0.5 ml sample extract to the top of the liquid chromatography column with a disposable pipette. The column is then sequentially eluted with the following eight solvent mixtures, each being collected in a separate vial.

(1) 25 ml 60/80 petroleum ether

(2) 25 ml 20% methylene chloride in 60/80 petroleum ether

(3) 25 ml 50% methylene chloride in 60/80 petroleum ether

(4) 25 ml methylene chloride

(5) 25 ml 5% methyl alcohol in methylene chloride

(6) 25 ml 20% methyl alcohol in methylene chloride

(7) 25 ml 50% methyl alcohol in methylene chloride

(8) 25 ml methyl alcohol.

Table 1 lists the classes of organic compounds which are expected to be found in each of the above eight fractions, if these species were originally present in the collected sample, together with their estimated minimum detection limits by infrared spectroscopy.

Each collected fraction is preliminarily reduced to about 0.25 ml using a Kuderna-Danish evaporator. The samples are then separately transferred to preweighed aluminum micro-weighing pans and the solvent allowed to evaporate in air. The weighing pans are reweighed as necessary until a marked decrease in the rate of weight loss indicates that the solvent is sufficiently removed to permit a reasonably accurate sample fraction weight determination to be made. The weights of each of the sample fractions should be tabulated.

In order to carry out infrared analysis, each of the samples is redissolved in a minimum quantity of methylene chloride and an infrared spectrum is obtained with a film of the sample placed between polished sodium chloride windows, following evaporation of the solvent. The infrared spectrometer should be a grating instrument. Many suitable models are available from a large number of manufacturers. Experience indicates that acceptable instruments for Level-1 analysis cost at least $6,000, examples are Perkin Elmer 457, Beckman IR-8, or an equivalent instrument

TABLE 1. CLASSES OF ORGANIC COMPOUNDS ELUTING IN EACH LIQUID CHROMATOGRAPHY FRACTION, AND THEIR APPROXIMATE IR DETECTION LIMITS

Fraction	Compound Type	Approximate IR Sensitivity*
1	Aliphatic hydrocarbons	1-10 μg
2	Aromatic hydrocarbons POM PCB Halides	1-10 μg
3	Esters Ethers Nitro compounds Epoxides	0.1-1 μg
4	Phenols Esters Ketones Aldehydes Phthalates	0.1-1 μg
5	Phenols Alcohols Phthalates Amines	0.1-1 μg
6	Amides Sulfonates Aliphatic acids Carboxylic acid salts	0.1-1 μg
7	Sulfonates Sulfoxides Sulfonic acids	0.1-1 μg
8	Sulfonic acids	0.1-1 μg

* Using Perkin Elmer 521 (or equivalent) when used by a professional IR Spectroscopist.

from any other manufacturer. The manufacturer's instructions for operating the infrared spectrometer should be carefully followed. No infrared absorbtion band should be less than 10 percent transmission (no greater than 90 percent absorbtion) for a satisfactory spectrum to be obtained.

The tabulated weights of each of the eight LC fractions, together with the IR spectra of each fraction should be transmitted to a Level 2 laboratory for appraisal, at the direction of the cognizant Project Officer.

ANALYTICAL COSTS

In view of the low level of analytical effort which is required in this analytical strategy, the analytical costs are accordingly modest and are tabulated below:

Sample extraction	$50
LC fractionations, 8 fractions	100
Solvent removal, weighing	100
Obtaining IR spectra, 8	180
Total Costs	$430

LEVEL 2 - QUALITATIVE AND QUANTITATIVE ANALYSIS

1. INTRODUCTION

The increasing awareness and concern regarding the emission of potentially hazardous organic materials from industrial processes, has resulted in a variety of analytical procedures being devised for their identification and quantification. Once a potential hazard is recognized, the cognizant Government agency can then implement emission measurement programs with an ultimate view to emission control. Chemical analysis of complex mixtures of unknown organic species is a challenging prospect for a knowledgeable and well equipped analyst. While opinions frequently differ regarding the choice of technique and the suitability of analytical methods for known pollutants, the possibility of unsuspected chemical hazards remaining undetected reiterates the need for a unified analytical approach which will maximize the probability that all chemical species will be detected and measured. This manual presents an optimal comprehensive analytical scheme for the measurement of organic compounds collected by established methods (see for example, Technical Manual for Process Sampling Strategies for Organic Materials - Monsanto Research Laboratory (1976).

(a) Rationale

In order that organic emission measurement programs may be carried out in a consistent, coordinated, and comprehensive manner, it is desirable that a moderately detailed procedural guide should be established for this purpose. This manual is intended for use by

experienced analytical chemists who have access to a wide range of state-of-the-art analytical techniques. It is not intended to consist of detailed step-by-step instructions, but rather will guide the user in a manner which is intended to ensure that the maximum of analytical data may be obtained from any complex organic mixture, irrespective of its source or method of collection.

In previous analytical studies of industrial emissions, various techniques have been used by different workers, with the result that differing data have sometimes been obtained for the same pollution sources. Furthermore, it is unfortunate that many techniques have previously been employed which have a limited capability for the determination of widely differing classes of organic compounds, with the result that much potentially valuable information relating to the emissions was not obtainable. It is not cost-effective to embark upon an analytical strategy for a complex emission source, without a reasonable probability that the maximum quantity of analytical data which is accessible to all modern state-of-the-art techniques will be obtained. If a unified and comprehensive analytical approach is not taken, much unnecessary cost and duplication of effort will be inevitable.

Thus, the ensuing analytical scheme has been developed with a view to meeting the analytical demands of any complex mixture of organic compounds. The high performance liquid chromatography (HPLC) separations proposed are equally suitable for a low molecular weight hydrocarbon, a high molecular weight ionic compound, or any other organic species falling between these extremes. HPLC separation techniques have a significantly superior efficiency to acid-base extraction methods. The qualitative and quantitative methods discussed include the most powerful techniques available

to the modern analytical chemist. Many species are more particularly suited to individual analytical techniques such as infrared spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry, and chromatography, but a combination of more than one of these techniques assisted by suitable dedicated computer routines constitutes a most powerful resource for the analytical chemist. It is envisaged that if the following analytical strategy is applied to any complex organic emission sample, the data obtained will represent the maximum information reasonably attainable by present organic analytical techniques.

(b) Overview

The initial step of the analytical scheme (See Figure 1) described in this manual consists of a sequential separation by HPLC resulting in a high resolution fractionation of complex mixtures of differing classes of organic compounds. It is anticipated that of the order of twenty or more HPLC fractions will be obtained for subsequent analysis.

The preliminary analysis will consist of screening each HPLC fraction by a combination of techniques such as infrared spectroscopy, nuclear magnetic resonance spectroscopy and high resolution mass spectrometry. Having thus identified the classes of organic compounds which are present in each of the fractions, qualitative and quantitative analysis will be carried out by gas chromatographic-mass spectrometry (GC-MS), gas chromatographic-infrared spectroscopy (GC-IR), gas chromatography (GC), nuclear magnetic resonance (NMR), and high performance liquid chromatography (HPLC). HPLC in combination with NMR, IR, and mass spectrometry (MS) will be utilized exclusively for identification and quantification of very polar

and ionic compounds, since these species are not amenable to separation by gas chromatography and have been largely ignored in earlier studies.

As can be seen in Figure 3, the separation into fractions and analytical screening will be iterative procedures, in so far as separation techniques will be reapplied as extensively as necessary until the screening techniques indicate that the complexity of each HPLC fraction is sufficiently reduced to facilitate more straightforward qualitative and quantitative analysis.

All of the techniques described in this manual have been available to the analyst for several years, and all of the equipment discussed is commercially available. The most recent analytical innovations are discussed where appropriate, and alternative methods are suggested only when these represent effective alternatives.

The final section of the manual presents an estimated cost breakdown for each phase of the analysis.

2. SAMPLE EXTRACTION AND RECOVERY

In most instances, samples of organic effluent will have been collected by means of a sampling train which may consist of various combinations of filters, cyclones, impactors, and porous polymer adsorbent traps. However, samples may also be provided which consist of liquids or granular solids other than particulate from impactors or cyclones. Additionally, solvent rinses from probes and filter holders may be provided for analysis; it is recommended that any such solvent rinses should be made with solvents such as methyl alcohol followed by methylene chloride,

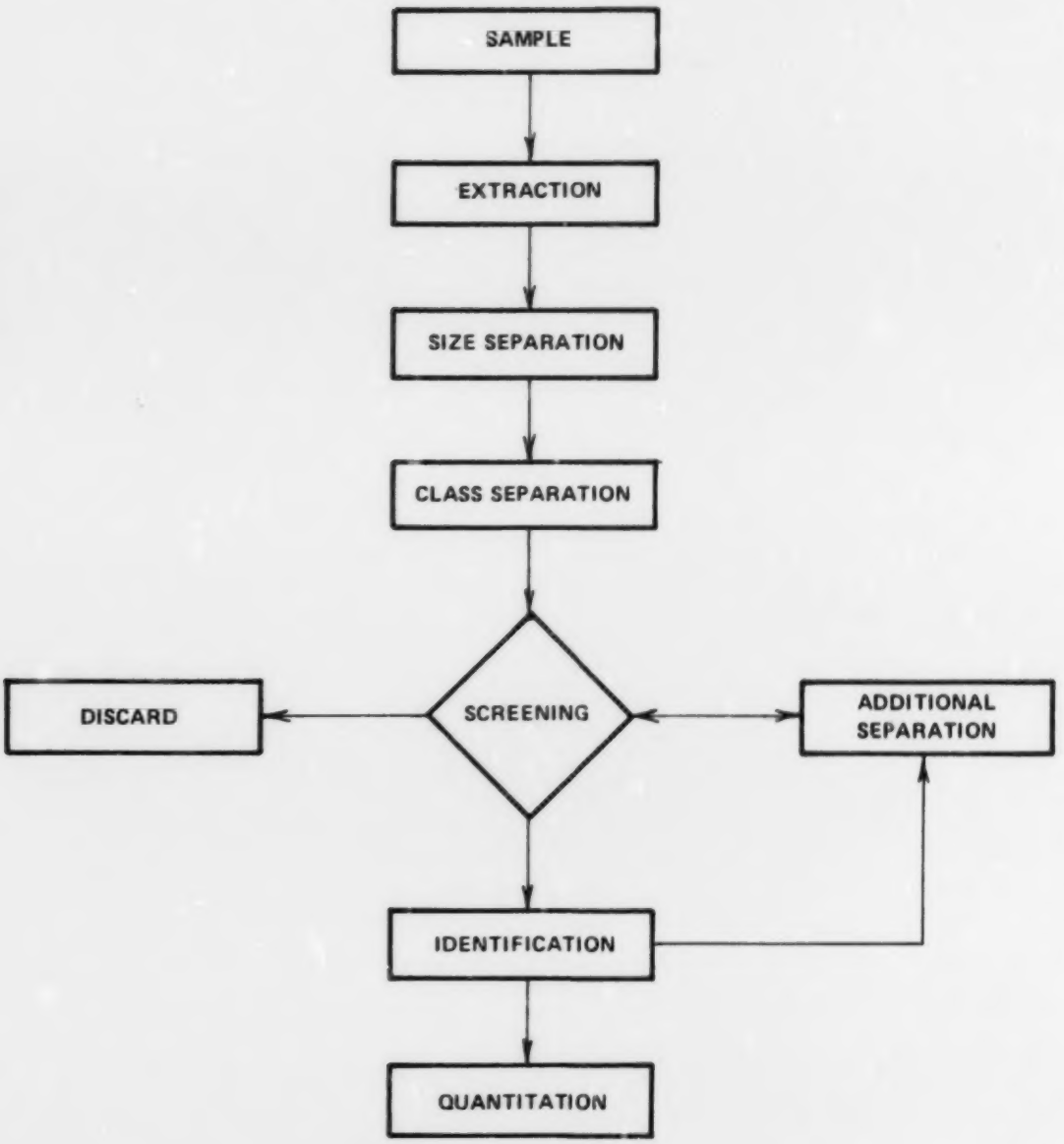


FIGURE 3. OVERALL ANALYTICAL STRATEGY

in order to ensure complete recovery of organic species. Procedures for sample extraction, and volume reduction are described below.

(a) Filters

Extraction of filter material should be carried out initially with methylene chloride followed by a reextraction of the residue and filter with methyl alcohol in order to ensure that all polar and non polar organic material is efficiently recovered. Extraction may be carried out using a Soxhlet apparatus for 24 hours, or by ultrasonic agitation of the filter with solvent in a sealed glass container for one hour, followed by conventional filtration of the shredded filter and insoluble particulate matter. We have found both methods of extraction to be satisfactory, although recent reports (1) suggest that ultrasonic agitation may be slightly superior.

(b) Cyclones, Impactors, and Probe Rinse

Particulate material which has been removed from a cyclone, impactor or rinsed from a probe may be solvent extracted in a similar manner to a filter. Soxhlet extraction may be carried out in a cellulose thimble plugged with glass wool, or in a fritted (fine) glass thimble with a glass wool plug. Ultrasonic extraction of particulate may be carried out by agitation with solvent in a sealed glass container followed by conventional filtration to remove the insoluble material.

(c) Porous Polymer Adsorbent Traps

Porous polymer adsorbent traps are coming into widespread use as a replacement for impinger collection in EPA Method 5 type sampling trains on account of their significantly higher efficiency. A typical adsorbent trap

is shown in Figure 1, which schematically depicts the Battelle Adsorbent Sampler (2,3). Collected sample may be recovered from these sampling devices by thermal desorbtion (4,5) or solvent extraction (2,3).

The preferred method of solvent extraction involves continuous solvent extraction of the adsorbent for a period of 24 hours (2,3). Such an extraction apparatus is shown in Figure 2, which depicts the continuous extraction assembly developed for the Battelle Adsorbent Sampler; the condenser and solvent reservoir are not shown. We would recommend that a similar apparatus should be used for the extraction of all porous polymer adsorbent traps.

The choice of solvent for extracting a porous polymer adsorbent trap depends partly upon the nature of the adsorbent. It is important that the solvent chosen does not affect the adsorbent in any way, yet is still an effective solvent for the removal of collected material. For porous polymer adsorbent traps utilizing Tenax, we would recommend extracting with a hydrocarbon such as pentane, since more polar solvents readily dissolve the adsorbent. The disadvantage of using a relatively inefficient solvent such as pentane is readily overcome by using the continuous extraction mode, when even polar compounds are extracted by pentane. Care should however be taken to ensure that the very polar compounds are extracted; such compounds may be anticipated on the basis of Level-1 studies, or from prior knowledge of the source. It is possible that extraction with a more polar solvent compatible with the adsorbent material may be required.

(d) Granular Solids

Granular solids should be crushed and ground to mesh size 200 or finer and then subjected to ultrasonic solvent extraction in the same manner as particulate samples. Alternatively the solid material should be suspended in the extraction solvent and subjected to high frequency dispersion (6).

(e) Liquids

Aqueous solutions may be solvent extracted by means of conventional liquid-liquid extraction in a separatory funnel; methylene chloride will frequently prove to be a suitable solvent. Alternatively when the organic compounds sought are believed to have a reasonable vapor pressure (most compounds except the very polar), vapor displacement and adsorbtion may be used. Inert gas is bubbled through the liquid, and is then passed through a trap containing chromatographic adsorbent where the volatilized vapors are trapped (7). Sample recovery from the adsorbent may be made by extracting it with a suitable solvent, as discussed previously.

A further aqueous extraction procedure involves percolating the solution through a column of chromatographic adsorbent or resin (8), again followed by solvent extraction or elution of the solid to recover the organic sample.

Organic oils should not be extracted but should be directly subjected to high performance liquid chromatography as described in the next section.

(f) Preparation of Extracts for Analysis

The manner in which the various sample extracts are combined depends upon the information that is required from the emission source. It is possible that particulate and vapor may need to be analyzed separately on account of data

required for an emission control strategy, for example. In any case, each extract or combination of extracts should be reduced to a volume of about 0.5 ml by a Kuderna-Danish evaporator, the sample is then ready for separation into fractions by high performance liquid chromatography. While it is true that some of the more volatile species may be partly lost during this procedure, the sampling techniques utilized for organic materials from process streams are generally not effective for the most volatile species in any case. Much of the sample loss is likely to be volatile hydrocarbons, which are generally regarded as less important from a health effect point of view.

It is usually desirable to add the prerequisite internal standards to the sample extract before volume reduction and liquid chromatography separation (3). However, the analysis of a grossly complex mixture as envisioned here precludes addition of internal standards to the HPLC fractions until qualitative analysis has been carried out, since it is impossible to prejudge the nature of the compounds present. The present analytical scheme incorporating high performance liquid chromatography separation techniques does however offer the significant advantage of truly quantitative sample fractionation. This largely obviates the usual necessity of adding internal standards before sample fractionation, since in the GPC and HPLC separations described, the sample integrity is expected to remain above 90%, whereas with the older acid-base-neutral fractionation techniques it was not surprising to lose well over 50% of the sample during the extraction procedures.

HPLC fractionation steps are not expected to introduce an uncertainty of greater than $\pm$10%, and thus when utilizing the quantification techniques described in Chapter 6, a high degree of analytical accuracy is expected to be achieved for even complex mixtures of organic compounds.

3. SEPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In order to successfully separate and analyze a complex mixture, the use of multiple chromatographic techniques, or sequential analysis, is necessary (Figure 4). The use of high performance liquid chromatography (HPLC) allows sequential analysis to be performed more quickly and efficiently than conventional column chromatographic techniques.

Significant improvements have come to liquid chromatography over the past ten years both in terms of column design and instrumentation. Presently, small diameter columns (2-5 mm) packed with supports of particle diameters down to 5 μ are being used. The eluent is pumped through the column at higher linear velocities than classical liquid chromatography (0.1 to 5 cm/sec), which results in larger pressure drops (200-5000 psi) across the column. This has resulted in efficiencies 100-1000 times higher than in classical methods, and thus the name, high performance liquid chromatography.

For this reason, high performance liquid chromatography is rapidly replacing older column and thin layer chromatographic techniques, and has taken a position alongside gas chromatography as a highly efficient, highly sensitive separation tool. Chromatographic columns with greater than 20,000 theoretical plates per meter are currently being used to affect high resolution separations.

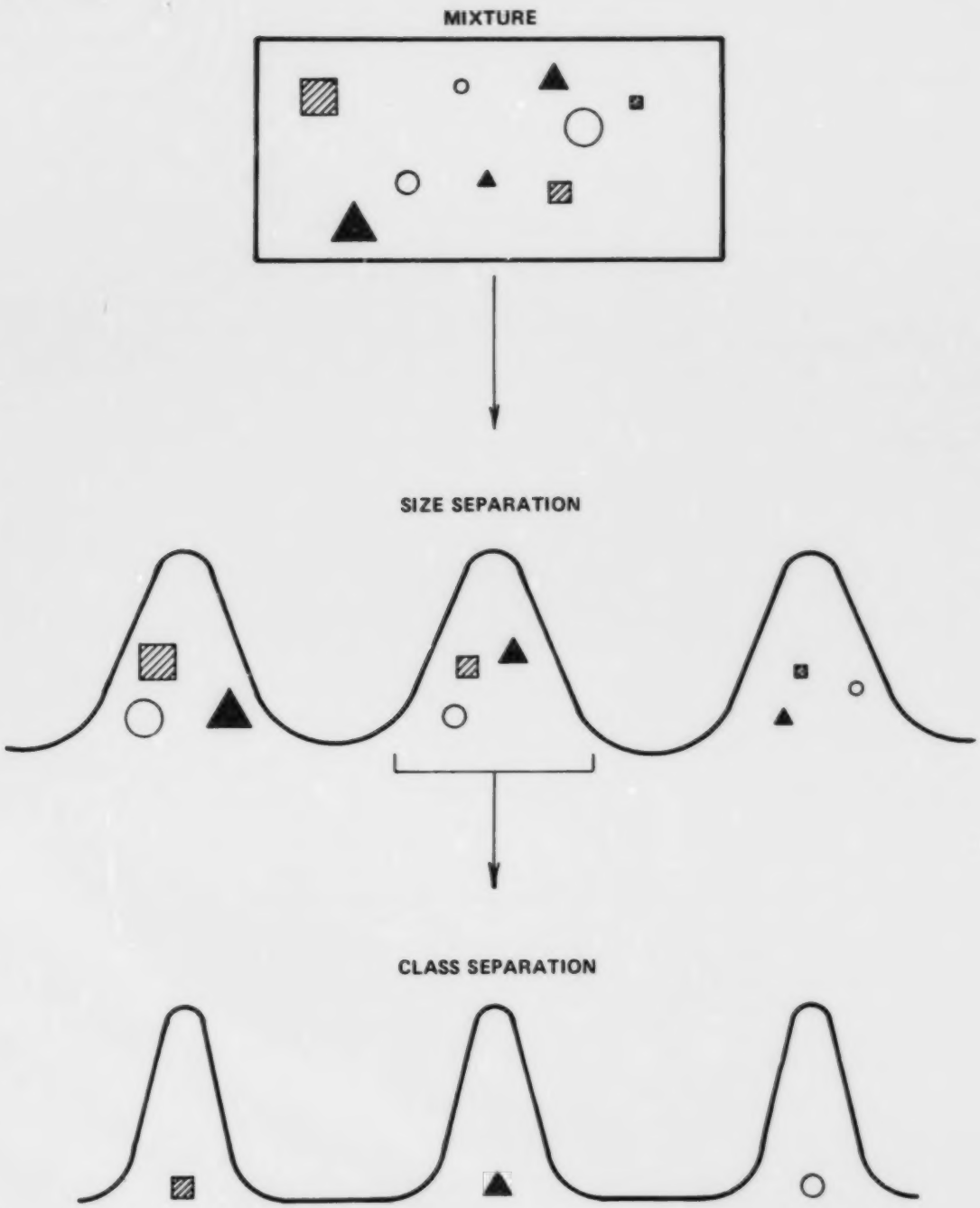


FIGURE 4. SEQUENTIAL ANALYSIS BY HPLC

As a result of the significant improvement in chromatographic columns, the ancilliary equipment required for their success has necessarily become more sophisticated. Although the foundation of high performance liquid chromatography has as its base the improvement in the conventional liquid chromatographic column, its overall success depends on precision instrumentation, such as constant flow, high pressure pumping systems, and detectors with micro-flowthrough cells to prevent remixing of resolved peaks. For a further discussion of high performance liquid chromatography, the following books (9-12) and review articles (13,14) should be consulted.

a) Separation by Size

The first step in the separation of a complex mixture is to separate by size or molecular weight, using gel permeation chromatography (GPC). Gel permeation chromatography is an exclusion technique in which retention is based on a molecule's ability to penetrate the pores of the chromatography support. Figure 5 illustrates the principles of gel permeation chromatography. Large molecules elute with the solvent front while small molecules totally permeate the support and elute later on. Within the range of elution volume, molecules can selectively permeate the support and thus be separated. Supports must be chosen for the exclusion limits that

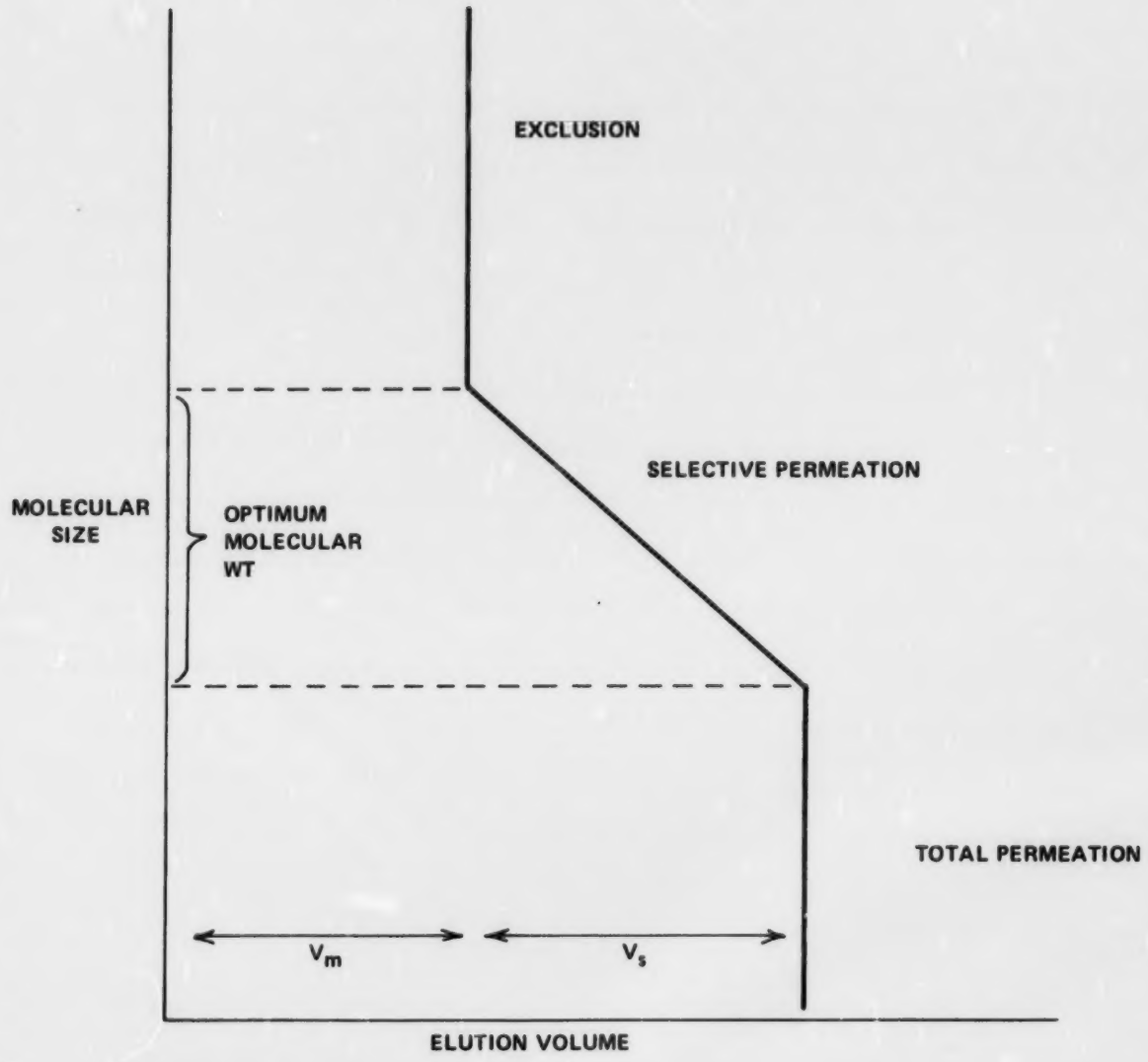


FIGURE 5. PRINCIPLES OF GPC

correspond to the molecular weight range that one desires to separate. For more detailed information, the following books and review articles should be consulted (9,12,15).

There are several advantages in using GPC as an initial separation method. A knowledge of the molecular weight of an individual fraction can dictate the choice of further separation steps such as gas or liquid chromatography as discussed subsequently. Narrow molecular weight ranges also allow simpler interpretation of the class separations that follow. Finally, information concerning molecular weight is useful for final identification purposes.

Figure 6 shows a fractionation by GPC which arbitrarily assumes that all the compounds of molecular weight under 500 are of interest. The number of fractions actually obtained as well as the molecular weight range of each fraction will depend on the individual samples analyzed. Since we are assuming the most complex possible case, all fractions will be further analyzed. With modern high performance liquid chromatographic columns, molecular weight differences of 15 percent can be distinguished and therefore the number of fractions collected during the first separation depends on the sample and the analysis goals.

(i) Column Packings

Separation in gel permeation chromatography is controlled by the type of packing used. The region of selective permeation depends

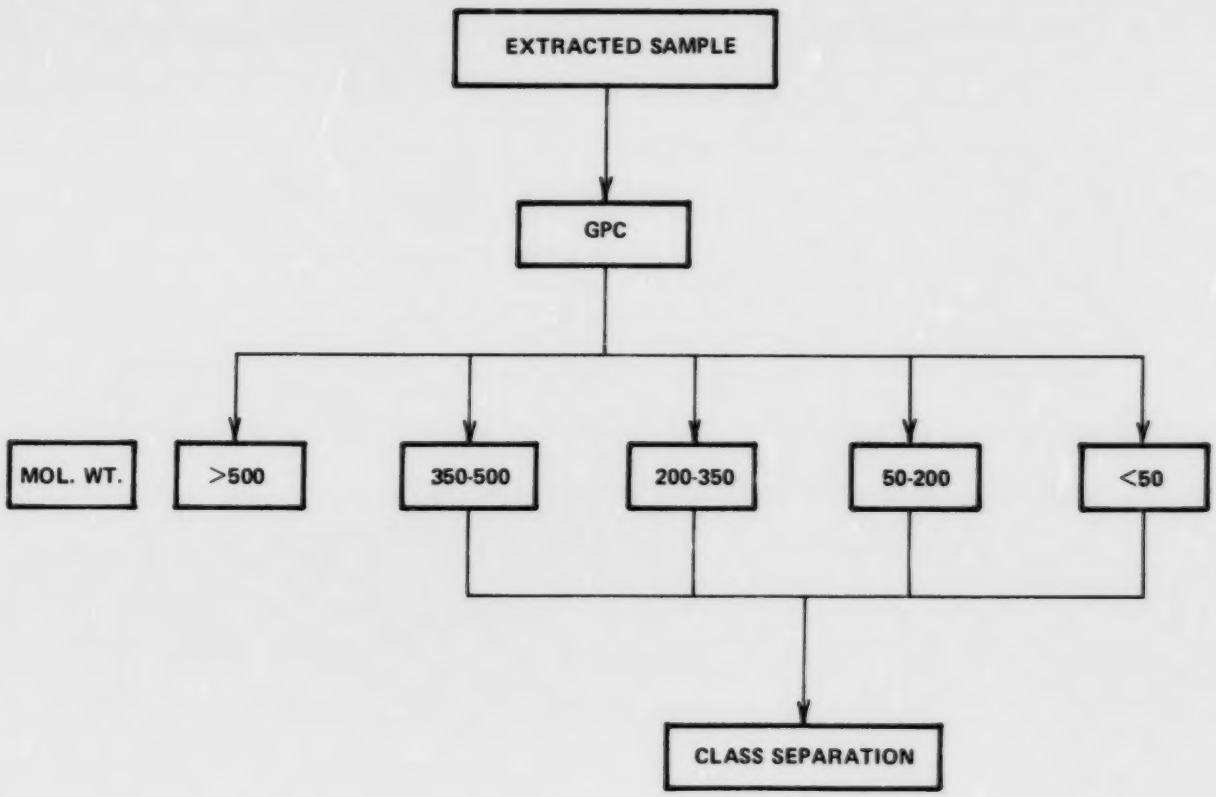


FIGURE 6. SEQUENTIAL SEPARATION – GPC

on the pore size of the support and therefore the choice of the packing material for a given separation is most important. Since we are interested in the selective permeation of small molecules (molecular weights under 500) supports with small pore sizes (100-500 A) should be used. The GPC packing most often used for sequential analysis is the semi-rigid type and is based on a styrene-divinyl benzene polymer (Styrogel, Biobeads, or equivalent). For complex mixtures, small particle GPC columns packed with micro Styrogel can be obtained with efficiencies of 10,000 plates per meter allowing the separation of compounds with differences of only 15 percent in molecular weight. For a good, although somewhat dated review of GPC packing materials, see Reference 16.

When using packings of the styrene-divinylbenzene type, the columns should not be allowed to dry out since channeling will occur which is deleterious to column efficiency. For the same reason, air bubbles should be avoided when connecting and disconnecting columns.

(ii) Mobile Phases

The mobile phase in GPC does not participate in the separation process. Therefore it should be chosen for its ability to dissolve the sample, its low viscosity, and its compatibility with the support and the detection system.

High viscosity solvents should be avoided since diffusion of the solutes is restricted and resolution is decreased. Some solvents cause excess swelling which is deleterious to the support material; acetone and alcohols are not used with styrene-divinylbenzene packings for this reason.

When choosing solvents for GPC, the detection system must always be considered. If UV is used the solvents must be transparent at the wavelength chosen. When refractive index detection is used, solvents with low refractive index allow more sensitive detection. Since air can be detrimental to the packing materials, the solvents chosen should always be degassed before using. Common solvents used for GPC include tetrahydrofuran, toluene, or methylene chloride.

(iii) Temperature

GPC is often run at elevated temperatures. High temperatures allow increased sample solubility and lowers the solvent viscosity. Since low molecular weight compounds are of interest, elevated temperatures are not expected to be necessary.

(iv) Sample Size

Sample size in GPC is limited mainly by sample viscosity and volume. A rough guide is that the sample solution should have a viscosity no greater than twice that of the mobile phase. With samples of low molecular weight, usually about 20 mg of sample per 100 ml of column volume can be injected.

(v) Column Calibration

Most GPC calibrations are based on units of molecular length or molecular hydrodynamic diameter, since size is related to the hydrodynamic volume of the molecules (15). For our purposes, a rough molecular weight calibration is sufficient. This can be done by measuring the elution volumes of a series of known compounds (preferably compounds of interest) within the molecular weight range of interest. This procedure

will define the limits of exclusion and permeation and also indicate approximate regions of molecular weight.

Gel permeation chromatography is the simplest form of high performance liquid chromatography. Its use will divide a complex multi-component sample into manageable fractions. Once Step 1 of sequential analysis is completed, samples can now be further separated by class.

b) Separation by Class

In the previous section, we have seen how narrow molecular weight fractions could be obtained from a complex mixture by separation on GPC columns. The GPC fractions can now be further separated according to polarity by bonded phase liquid chromatography.

Bonded phase liquid chromatography is a form of liquid partition chromatography in which the stationary liquid is permanently chemically bonded to the support. Although the mechanism of retention is no longer strictly partition, bonded phases possess distinct advantages over liquid-liquid systems. Since the stationary phase is bonded to the support, it cannot be stripped off by the eluent and therefore precolumns are not necessary. More importantly, gradient elution, an extremely important technique which will be discussed shortly, can be performed with a wide choice of solvents. Finally, a wide range of selectivity can be obtained by the proper choice of chemically bonded phase.

In this second step of sequential analysis, a reverse phase bonded support should be used. It has been shown that for similar solutes,

a plot of the log of retention time versus water solubility yields a straight line, when using reverse phase columns with water-methanol mixtures as the mobile phase (17). Since we have already obtained fractions of narrow molecular weight, it can be assumed that retention will be roughly a function the particular functional groups attached to the molecule. We can therefore obtain a rough class separation by performing gradient elution on a high performance reverse phase column.

(i) Gradient Elution

Since individual GPC fractions will usually contain components with widely different chemical structures, isocratic solvent conditions (constant strength) cannot be employed for the elution of such mixtures. Since the relative migration rates of individual components in the mixture will vary widely, early eluting peaks are poorly resolved while excessively retained peaks require long analysis times. Therefore the technique of gradient elution would be used extensively during the class separations.

In gradient elution, the mobile phase composition is continuously changed from a weak to a strong eluent during a chromatographic run. In this way, compounds of widely different polarity can be eluted from the column in a reasonable length of time. Quantitative recovery of the injected sample is therefore possible when a strong enough eluent is used. As we shall see, this technique allows a total class separation of widely different compounds.

The instrumentation used to perform gradient elution can vary widely. Modern liquid chromatography usually employs two high pressure pumps which meter solvents from their reservoirs into a mixing chamber under pressure and to the chromatographic column.

Solvent composition can thus be changed continuously during a chromatographic run by differentially varying the flow rates of the two pumps. This can be accomplished electronically with the use of a gradient programmer. Since the flow rates of the two pumps can be precisely controlled, reproducible gradients can be formed giving retention times with precisions less than 1 percent relative standard deviation.

An important consideration when using gradient elution is column regeneration. After a chromatographic run, the mobile phase must be returned to initial conditions and reequilibrated with the stationary phase before attempting another run. Also the solvents chosen for gradient elution must be compatible with the detection systems used to monitor the eluent. This will be discussed in more detail in a later section. It is important to reemphasize the importance of gradient elution in the analysis of complex mixtures. This technique is the basis of the class separations to be discussed.

(ii) Column Packings

Many different column packing materials can be used to further develop a sequential analysis. Reverse phase packings are recommended

for the following reasons. Firstly as we have mentioned, a rough separation by class is possible using this support material. Secondly, one gradient run allows the elution of a wide range of compounds from ionic and very polar to nonpolar species. This means that the entire sample can be eluted from the column without losses. Silica gel, which has been used previously as a second column in sequential analysis, suffers in this regard. Finally, column reequilibration after a gradient run is extremely fast using reverse phase packings. Since we are interested in obtaining as much resolution as possible, columns containing microparticle (5-10 μ) reverse phase packing should be chosen. These columns can generate about 10,000 plates per meter, and are available from a variety of different manufacturers. When large samples are to be chromatographed preparative columns should be used. These columns are capable of handling over one gram of material without overloading.

(iii) Mobile Phases

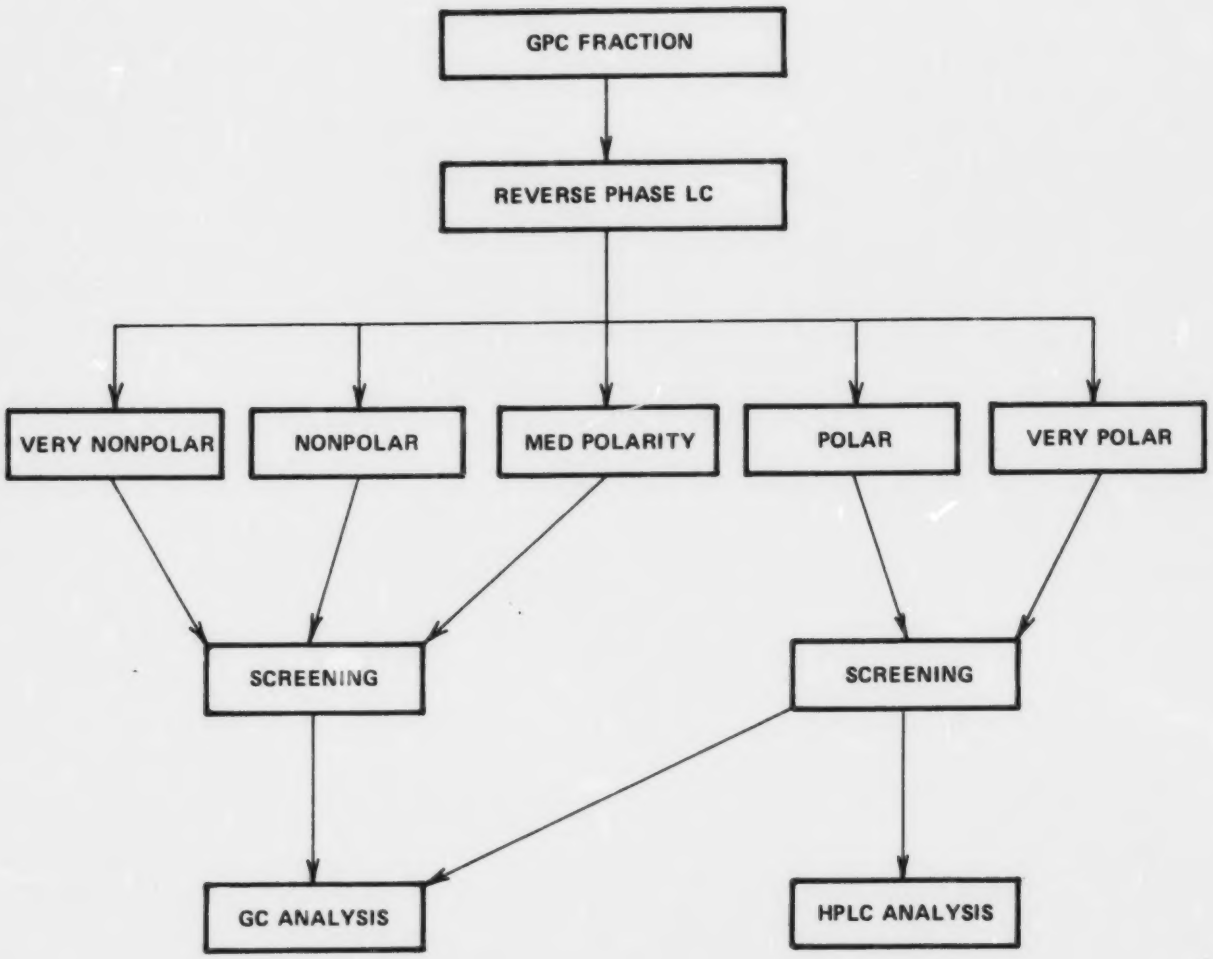
Typical gradient solvents in reverse phase chromatography are water modified with methanol, isopropanol, or acetonitrile. The choice of solvents is dictated by the type of detection used. When using refractive index detection, gradient elution is difficult, but not impossible, to run since large changes in baseline occur. There is usually no problem using a UV detector. Detection will be discussed further in the next section.

(iv) Temperature

Elevated temperatures are sometimes used in reverse phase HPLC, since lower viscosities and therefore higher efficiencies can be obtained. Retentions are usually lowered at higher temperatures. With the use of high performance micro-particle columns, elevated temperatures are not necessary on a routine basis, but certainly should be used if the added efficiency can solve a particular separation problem.

(v) Functional Group Separation

The fractions obtained from GPC can be further separated by reverse phase HPLC using gradient elution. Figure 7 shows, once again, a rough guide to the kind of separation one might expect. Very polar compounds will elute first and very nonpolar compounds will elute last. The fractions which are collected during this run may contain 1 or 2 components in a simple case or 100 components in an extremely complex mixture. However, these fractions should be sufficiently homogeneous to be screened by spectroscopic techniques (see Chapter 4) for classes of organic compounds. If screening techniques indicate that a fraction contains compounds amenable to GC analysis, this route should be taken. Complex mixtures or mixtures containing compounds which cannot be run by GC should be further separated by HPLC. The choice of columns for further separation by HPLC is discussed in Chapter 5. Figure 8 is an illustration of the wide variety of compounds that can be eluted during



FIGU E 7. SEQUENTIAL SEPARATION – REVERSE PHASE HPLC

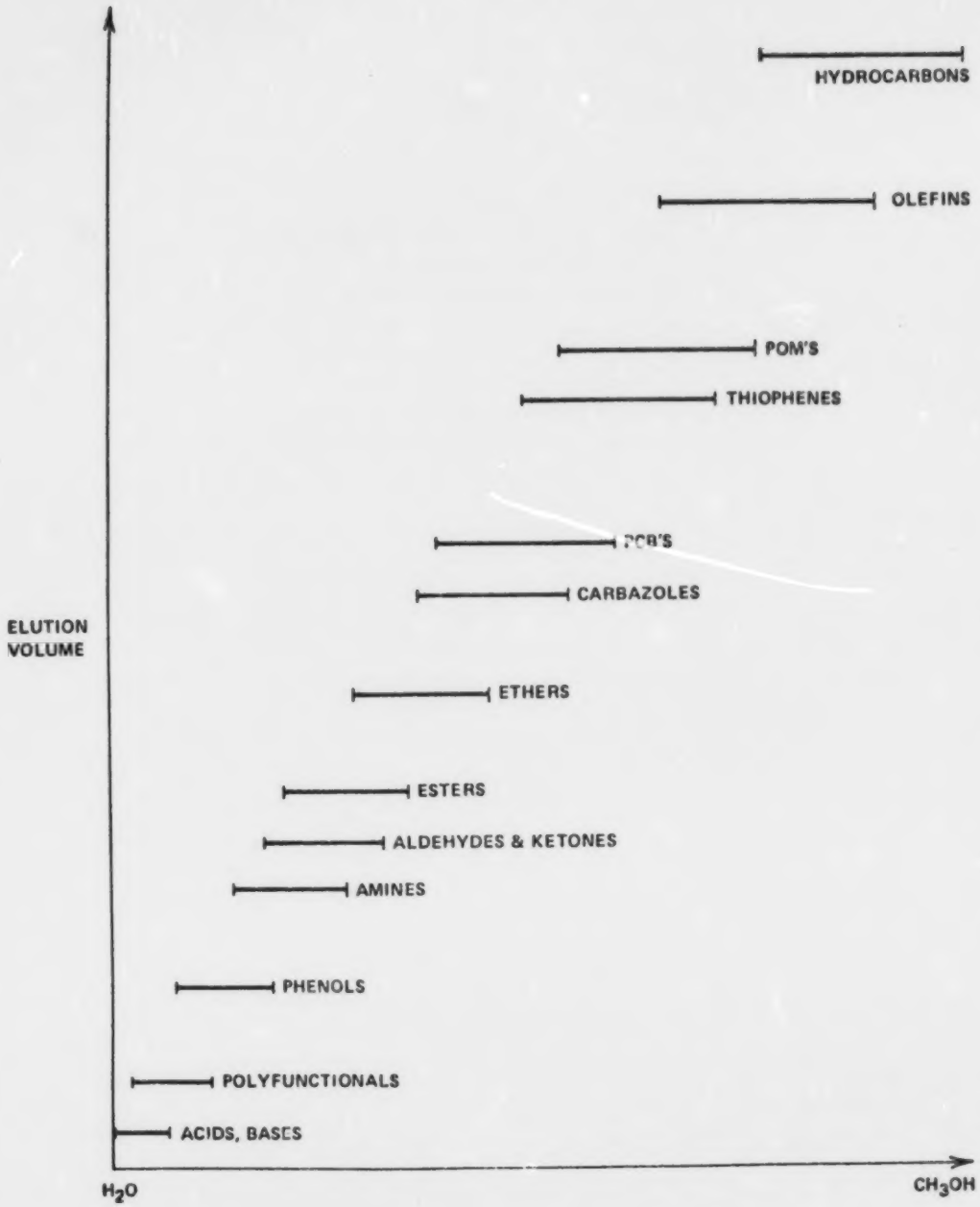


FIGURE 8. TYPICAL ELUTION ORDER BY CLASS ON REVERSE PHASE HPLC

a gradient run from water to methanol. Large ranges in solvent composition are given to account for the molecular weight differences in a given class of compounds. For example, naphthalene and coronene might be expected to elute towards the two extremes of the POM range. Relative retentions for standard compounds of interest should be determined prior to unknown analysis so that narrow fractions can be obtained from the gradient run.

After step 2 of sequential analysis a considerable separation of the samples has been accomplished and effective screening techniques should now be used to determine further cause of action.

c) Detection Systems

The ability to continuously monitor the column effluent is yet another advantage of modern HPLC. With the advent of small volume (8-20 μl) flow-through cells, highly resolved chromatographic peaks can be detected without significant remixing in the detector cell. Both universal, or bulk property, detectors and specific, or solute property, detectors are presently available, but unfortunately, there is no HPLC equivalent to the flame ionization detector in GC for both universal and sensitive detection. Nonetheless, valuable information can be obtained by HPLC detection methods and frequently quantitation can be achieved.

The two universal detectors in common use are the refractive index detector and the solute transport detector. The refractive index (RI) detector measures the difference in refractive index between the

mobile phase and the mobile phase containing dissolved solute. Since it responds to all sample components it is often the choice when non UV active compounds are analyzed. Its major limitation is low sensitivity, and mobile phases should be chosen to enhance differences in refractive index between solvent and solutes. Gradient elution is difficult to perform using RI detection since large baseline changes occur when the mobile phase is changed. Refractive index matching of the initial and final solvents is essential if gradient elution is to be performed. This can be accomplished when reverse phase chromatography is being used (class separation) by an appropriate mixture of methanol and acetonitrile to match the refractive index of water. RI detectors have a large linear range but unfortunately lower limits of detection are about 1 μ gram for favorable solutes.

The second universal detector used in HPLC is the solute transport, or moving wire detector. The chromatographic eluent is dripped over a moving wire depositing some sample. The wire is then fed to an oven which evaporates the mobile phase, and then to a flame ionization detector (FID). The advantages of this system are twofold. Detection is not dependent on the solvent and thus gradient elution can be used, and secondly, the sensitivity is potentially better than RI detection. However, the detector is limited to relatively nonvolatile solutes, and since this is a destructive detection technique collection and further analysis of the sample is impossible. Finally, although FID is a sensitive detection

device, only about 1-2 percent of the sample to be analyzed is deposited on the wire and therefore overall detection at present is only slightly better than RI. For these reasons RI detection is recommended as a universal detector for the present analytical strategy.

The two specific detectors most commonly used are UV and fluorescence. UV detection is the most widely used in HPLC today, and is the first detection choice for compounds that absorb in the UV. High sensitivity and specificty is obtained using UV detection and lower limits of detection for favored samples is in the nanogram range. The first detectors used a mercury lamp and monitored the eluent at 354 nm, but more recently multiwavelength detectors have become available in which eluent monitoring from 200-800 nm can be obtained. Although multiwavelength detectors suffer a slight loss in sensitivity, the increased specificity and ability to monitor wavelength maxima of compounds make them valuable detection systems.

The second specific detector used for HPLC monitoring is the fluorescence detector, which is receiving a great deal of interest recently due to its high sensitivity and high selectivity. Fluorescent compounds can be detected and quantitated in the presence of coeluting compounds. As an example of its usefulness in the analysis of organic eluents, POM's may be selectively detected during the class separation. A combination UV, fluorescence detector is presently commercially available and could prove valuable in monitoring organic effluents during sequential analysis. When using fluorescence detection, care should be taken to degass the solvents used for the mobile phase and to avoid halogenated solvents, since these conditions quench fluorescence.

For the analysis of a complex multicomponent mixture, a combination of detectors in series is an effective way to monitor the HPLC eluent. Valuable information about individual fractions or peaks can be obtained and correlated with the screening techniques used in Chapter 4 for compound or functional group identification. Furthermore, quantitative analysis can be performed as discussed in more detail in Chapter 6.

We have referred more than once to the importance of solvent detector compatibility; it is important to emphasize this point again, since transparent solvents must be used with specific detectors and if RI detection is used to monitor a gradient, refractive index matching is essential.

4. SPECTROSCOPIC SCREENING TECHNIQUES

a) Introduction

Separation of the sample into organic classes by HPLC will provide fractions which can be screened for complexity and/or types of compound which are present. Identification of individual components is not intended in a screening technique, but rather the ability to discern the presence or absence of interferring compounds in a given class, and to judge the overall complexity of the mixture. In the case of the most complex mixture, screening will provide an indication of the type of separation needed to further fractionate the sample. For simple mixtures, screening will be a step toward qualitative and quantitative analysis.

The techniques used for screening include infrared spectroscopy, nuclear magnetic resonance spectroscopy, and high resolution mass spectrometry.

4 b) Nuclear Magnetic Resonance Spectroscopy

(1) NMR as a Screening Technique

Nuclear magnetic resonance spectroscopy (NMR) is a powerful screening technique for mixtures of organic compounds. Although not used extensively in the past for analysis of mixtures, recent progress in instrumentation to improve resolution, stability, and sensitivity has made the observation of components of a mixture practical. The types and relative amounts of various functional groups in the mixture can be determined, and types of hydrocarbons can be identified. As a screening technique, NMR not only characterizes the general nature of the fraction, but also provides sufficient information to determine the types of further separations required. For

example, a typical liquid chromatography fraction submitted for screening might be expected to contain only polynuclear aromatic hydrocarbons. Their presence can immediately be confirmed by observation of the aromatic region of the spectrum. Other compounds with similar polarity eluted at the same time would also be observed, and can be classified as to functionality. Since the types of compounds to be separated are identified, a scheme for their separation can be devised. Thus, obtaining the NMR spectrum of a sample has become a logical step in the analysis of organic components of a mixture.

The presence of various functional groups and identification of the types of hydrocarbons is determined from the chemical shift of peaks in the spectrum. This is due to the fundamental principle of NMR that the same nucleus in a different chemical environment resonates at a different frequency. A partial list of the approximate chemical shifts for protons in various functional groups is provided in Table 2. A more comprehensive listing of chemical shifts for protons and for other nuclei as well as a general review of NMR in the analysis of organic compounds is available from a number of sources (18-22).

The recent advances in instrumentation have extended NMR such that less abundant nuclei can now be observed. The greatest growth has been in ^{13}C NMR. Carbon is of extreme importance due to its fundamental role in the structure of organic compounds. The carbon backbone of a compound and nonprotonated functional groups containing carbon, such as carbonyls or nitriles, can now be observed directly. Proton NMR and ^{13}C NMR together cover almost all organic compounds, making the combined methods a powerful screening technique for mixtures of organic components.

TABLE 2. APPROXIMATE PROTON CHEMICAL SHIFTS OF REPRESENTATIVE FUNCTIONAL GROUPS

Type of Proton		Chemical Shift[(a)]
Cyclopropane		0.2
Primary	RCH_3	0.9
Secondary	R_2CH_2	1.3
Tertiary	R_3CH	1.5
Vinylic	C=C-H	4.6-5.9
Acetylenic	C≡C-H	2-3
Aromatic	Ar-H	6-8.5
Benzylic	Ar-C-H	2.2-3
Allylic	C=C-CH_3	1.7
Fluorides	HC-F	4-4.5
Chlorides	HC-Cl	3-4
Bromides	HC-Br	2.5-4
Iodides	HC-I	2-4
Alcohols	HC-OH	3.4-4
Ethers	HC-OR	3.3-4
Esters	RCOO-CH	3.7-4.1
Esters	HC-COOR	2-2.2
Acids	HC-COOH	2-2.6
Carbonyl cmpds.	HC-C=O	2-2.7
Aldehydic	RCHO	9-10
Hydroxylic	ROH	1-5.5
Phenolic	ArOH	4-12
Enolic	C=C-OH	15-17
Carboxylic	RCOOH	10.5-12
Amino	RNH_2	1-5
gem-dichlorides	HCl_2	5.8

(a) ppm from TMS.

^{13}C NMR has an additional advantage over proton NMR. Since the shift range for carbon is 600 ppm compared to less than 20 ppm for proton nuclei, there is an enhancement in effective resolution. Broad envelopes of overlapping peaks in proton NMR may many times be resolved into individual carbon resonances. This allows for ready identification of specific functional groups. For a review and further discussion of ^{13}C NMR see references 23-26.

(ii) Limitations of NMR in a Screening Technique

The primary limitation of NMR is sample size. For conventional continuous wave (CW) NMR, milligram quantities are needed for proton NMR, and natural abundance ^{13}C NMR is essentially impossible. This has largely been overcome by the use of Fourier Transform NMR. Unlike CW NMR, which slowly sweeps the radio frequency (RF) applied in a fixed magnetic field, Fourier Transform (FT) methods use short bursts of RF power at a discrete frequency for rapid data acquisition. The observation of spectra is made after the RF power is turned off. In this mode, data are generated as free induction decay patterns (signal intensity versus time). The Fourier transform of this is then mathematically determined, which is a conventional NMR spectrum (signal intensity versus frequency). This multichannel excitation and detection results in a hundredfold improvement in sensitivity. For proton NMR in the pulse Fourier transform mode, a practical minimum is 10 μg of sample. For ^{13}C NMR, milligram quantities are required. Spectra of smaller amounts may be obtained by using micro-techniques discussed later. The availability of a dedicated

computer also provides the opportunity for data handling such as spectral arithmetic. The spectra can be adjusted in size and unwanted components can be removed by subtraction without the necessity of chemical separation. For a detailed discussion of FT-NMR, see reference 27.

The only practical alternative to operating in the Fourier transform mode is to operate in the continuous wave mode while using a time averaging computer (computer of average transients, CAT). Even here, unavoidable instability of the system sets the upper limit over which signal averaging can be carried out without loss of effectiveness or degradation of resolution to 20-30 hours. Since the signal to noise ratio (S/N) increases with only the square root of the number of scans, the time required per sample to achieve a suitable S/N is approximately two orders of magnitude greater than time averaging of FT scans. This would be prohibitive due to both time and cost unless an unusually large amount of sample is available.

Another limitation of NMR is interference from solvent peaks. Large solvent peaks may not only obscure regions of interest, but also degrade the general quality of the spectra. This is due to introduction of spurious resonances (beat frequencies) into the spectrum, difficulties in phasing the spectrum, and loss of accuracy due to limitations of analog-to-digital conversion. Several techniques have been developed to suppress an unwanted solvent signal (28). However, for routine proton NMR, using an unprotonated solvent, such as CCl_4 or CS_2, or using a minimum amount of deuterated solvent for systems with few exchangeable

protons is usually satisfactory. This will limit the observation of those amines, acids, or other compounds with exchangeable hydrogens which have low solubility in unprotonated solvents.

(iii) Experimental Details

Preparation of Sample. Each sample fraction from HPLC is likely to be in a solvent unsuitable for NMR, such as protonated solvents. Should this be the case, the sample must first be separated from the extraction and separation solvents. Some of the methods which might be used include: lyophilization (freeze-drying); evaporation with a Kuderna-Danish concentrator; evaporation with a stream of inert gas; removal under a vacuum at room temperature. In any of these methods, the primary problem is the possibility of loss of volatile sample components. If heating is used there is also the possibility of decomposition. The actual choice of a method will depend on the nature of the solvent. For example, for more volatile solvents such as the hydrocarbons, evaporation using an inert gas (nitrogen, helium, argon) is preferable. For more polar, less volatile solvents, such as water, lyophilization is suggested. Removal of the solvent under a vacuum at room temperature should be avoided unless the components are known to be nonvolatile, and the solvent is unable to be removed adequately by other methods.

Choice of Solvent. After the separation solvents are removed (when needed), the sample is redissolved in a solvent suitable for NMR. The choice of a suitable solvent depends on a number of factors. It

must first of all be of appropriate polarity to redissolve the sample. This can be determined from the nature of the HPLC fraction and the elution solvent. The solvent must also allow a sufficient window for the observation of the sample. Ideally for proton NMR the solvent should contain no protons, and for carbon NMR it should contain no carbon, thus providing no solvent obstructions in the spectrum. Typical nonprotonated solvents include carbon tetrachloride and carbon disulfide. These compounds may not be desirable, however, due to low solubility of the sample fraction. Therefore, as a second choice, deuterated solvents may be used for proton NMR, and a solvent with one type of carbon may be used for carbon NMR. Deuterated solvents should be chosen such that the resonance of residual protons is outside the region of probable interest (See Table 3). If interferences are unavoidable, two solvents with differing residual resonances may be used in series to observe the entire spectrum. In any case, a minimum amount of solvent should be used to avoid a large solvent peak which can degrade the spectrum. Finally, the ease of removal of the solvent should be considered, since the sample may need to be further separated and characterized following the determination of the NMR spectrum. Solvents such as dimethylformamide, or dimethyl sulphoxide, which are very difficult to remove, should be avoided.

Standards. The chemical shifts for a given nucleus are extremely small compared to the resonance frequencies of the nuclei. While it is possible to measure frequencies in the megahertz range to 0.1 Hz, there

TABLE 3. CHEMICAL SHIFTS OF DEUTERATED SOLVENTS

Solvent	Chemical Shift[a] (mult)[b] Residual Protons	Carbons
Acetic Acid-d_4	11.53(1) 2.03(5)	178.4(br) 20.0(7)
Acetone-d_6	2.04(5)	206.0(13) 29.8(7)
Acetonitrile-d_3	1.93(5)	118.2(br) 1.3(7)
Benzene-d_6	7.15(br)	128.0(3)
Chloroform-d	7.24(1)	77.0(3)
Cyclohexane-d_{12}	1.38(br)	26.4(5)
Deuterium Oxide	4.63(DSS) 4.67(TSP)	
1,2-Dichloroethane-d_4	3.72(br)	43.6(5)
Diethyl-d_{10} Ether	3.34(m) 1.07(m)	65.3(5) 14.5(7)
Diglyme-d_{14}	3.49(br) 3.40(br) 3.22(5)	70.7(5) 70.0(5) 57.7(7)
Dimethylformamide-d_7	8.01(br) 2.91(5) 2.74(5)	162.7(3) 35.2(7) 30.1(7)
Dimethyl-d_6 Sulphoxide	2.49(5)	39.5(7)
p-Dioxane-d_8	3.53(m)	66.5(5)
Ethyl Alcohol-d_6 (anh)	5.19(1) 3.55(br) 1.11(m)	 56.8(5) 17.2(7)
Glyme-d_{10}	3.40(m) 3.22(5)	71.7(5) 57.8(7)
Hexafluoroacetone Deuterate	5.26(1)	122.5(4) 92.9(7)
HMPT-d_{18}	2.53(2 x 5)	35.8(7)
Methyl Alcohol-d_4	4.78(1) 3.30(5)	49.0(7)
Methylene Chloride-d_2	5.32(3)	53.8(5)
Nitrobenzene-d_5	8.11(br) 7.67(br) 7.50(br)	148.6(1) 134.8(3) 129.5(3) 123.5(3)
Nitromethane-d_3	4.33(5)	62.8(7)
Isopropyl Alcohol-d_8	5.12(1) 3.89(br) 1.10(br)	 62.9(3) 24.2(7)
Pyridine-d_5	8.71(br) 7.55(br) 7.19(br)	149.9(3) 135.5(3) 123.5(3)
Tetrahydrofuran-d_8	3.58(br) 1.73(br)	67.4(5) 25.3(br)
Toluene-d_8	7.09(m) 7.00(br) 6.98(m) 2.09(5)	137.5(1) 128.9(3) 128.0(3) 125.2(3) 20.4(7)
Trifluoroacetic Acid-d	11.50(1)	164.2(4) 116.6(4)
2,2,2-Trifluoroethyl Alcohol-d_3	5.02(1) 3.88(4 x 3)	126.3(4) 61.5(4 x 5)

(a) ppm relative to TMS.

(b) The multiplicity of the peak; br indicates a broad peak.

is no independent means to measure the magnetic field to the accuracy of 1 part in 10^8 or 10^9. Thus absolute measure of the resonance is impossible, so chemical shifts are reported relative to a standard or reference.

There are two types of references used in NMR: internal and external. An internal reference is a compound that is dissolved directly in the sample solution. The reference is uniformly distributed at a molecular level through the sample, such that the magnetic field acts equally on the sample and reference molecules. The only serious problem with an internal reference is the possibility of intermolecular interaction which would influence the resonance frequency of the reference. The most common reference, now generally accepted for both proton and ^{13}C, is tetramethylsilane (TMS). It is relatively inert, highly volatile, gives a single peak in both proton and carbon NMR, and resonates at a field higher than most common nuclei. TMS, however, is not soluble in aqueous solutions. The common reference compound for aqueous solutions is sodium 2,2-dimethyl-2-silapentane-5-sulfonate, $(CH_3)_3Si(CH_2)_3SO_3Na$, (DSS). This is used at a low concentration so the methyl singlet is observed but the spin coupled methylene groups do not interfere appreciably. Shifts measured with respect to TMS in chloroform will be within a few hundredths of a ppm for the same peaks measured with respect to DSS in water.

An external reference is a compound placed in a separate container from the sample. This may be in a sealed capillary tube inside

the sample tube, or in the annulus with the sample inside the capillary tube. When the sample is rotated rapidly the reference signal appears as a sharp line superimposed on the spectrum of the sample. An external reference removes the problems of intermolecular interaction, chemical reaction, and insolubility in the sample solution. However, there is a difference in the bulk magnetic susceptibility between sample and reference. The susceptibility correction is normally < 1 ppm. For nuclei with large chemical shifts, as ^{13}C, the correction may not be necessary. For proton resonances, when accurate chemical shifts are needed, a correction for the difference in susceptibility should be made. For dilute solutions of the sample, the conversion to internal TMS may be made by means of measured frequency differences for TMS in different solvents. (See Table 4). This is particularly crucial for the identification of specific compounds from reference spectra, but may be ignored for many spectra of mixtures if only general classification of the types of compounds is desired.

NMR data are usually measured in frequency units (hertz) from the chosen reference. However, the chemical shift is dependent on the value of the magnetic field. Therefore, it is customary to report chemical shifts in the dimensionless unit of parts per million (ppm), which is independent of the rf frequency or magnetic field strength. The chemical shift in ppm (δ) is

$$\delta = \frac{\nu s - \nu R}{\nu R} \times 10^6,$$

where νs and νR are the resonance frequencies of the sample and reference, respectively.

TABLE 4. RELATIVE RESONANCE FREQUENCIES FOR PROTON REFERENCE COMPOUNDS

Compound	Chemical Shift (ppm)
Internal TMS	0
TMS in CCl_4[a]	0.43
TMS in $CDCl_3$[a]	0.52
t-Butanol	1.39
Cyclohexane	1.63
Acetone	1.87
Dioxane	3.80
Water[b]	5.14
Benzene	6.95

(a) 1 percent by volume

(b) Temperature dependent

For this work, a dilute solution of TMS in carbon tetrachloride in the annulus of an NMR tube is recommended. This allows for the use of micro techniques as discussed in the next section and removes the problem of solubility in aqueous solutions. The minimum amount of TMS required to observe a peak should be used to avoid degrading the spectrum. All chemical shifts should be reported in ppm relative to TMS.

Micro Techniques. One method used routinely to improve sensitivity recommended for this work is the use of micro techniques. The goal is a small volume of sample at the maximum concentration such that the entire sample can be detected by the instrument. This can increase the effective sensitivity by as much as 100 times. Commercially available accessories include microcells, which confine a small amount of sample to the region of the receiver coil in the probe, and mini-probes, which are designed specifically for small sample volumes by using specially would receiver coils. Further details and instructions as to the use of these techniques can be found in reference 29 and the manufacturers' literature.

An effective microcell can also be easily constructed by sealing approximately 20 μl of the sample solution in a 2 mm O.D. capillary tube slightly longer than a standard NMR sample tube. The capillary tube is inserted into the sample tube and held in place by protruding through a tight-fitting hole in the tube cap. One or more

Teflon spacers around the capillary tube to hold it firmly in the center of the sample tube are also helpful. Before this technique is first used with a particular probe in a given instrument, the required amount of sample and the optimum position of the capillary tube must be determined. This is done by using a known compound in the capillary tube and adjusting the position of the tube and the sample amount until the resonance peak is maximized. To run a spectrum, a dilute solution of TMS (and lock compound if the instrument has an internal lock) in carbon tetrachloride is placed in the annulus. A solution of the sample is placed in the capillary tube.

In summary, nuclear magnetic resonance spectroscopy is an important tool for classifying mixture components. NMR readily identifies functional groups and estimates the complexity of a mixture. A combination of proton and carbon-13 NMR now allows observation of almost all organic compounds, making it applicable to all types of mixtures. The only major problem is sensitivity, which is to a large extent overcome by the use of Fourier transform NMR and micro-techniques.

4. c) Infrared Spectroscopy

(i) Introduction

Infrared spectroscopy has been, and still is, the most widely used tool for identification of organic compounds. One of the major uses of infrared spectroscopy (IR) has been during the separation of complex organic mixtures. It has provided functional group (especially on polar groups) identification in such mixtures, it has been used to monitor or screen the course of separation of the mixture, and ultimately it has been used to identify the compounds present when the separation has proceeded as far as required. As with most analytical tools, less information is obtainable when the mixture was very complex as compared to when the mixture was separated into smaller fractions. Now, however, the use of Fourier Transform infrared systems (FT-IR), has clearly demonstrated that a major increase in sample information can be obtained - even on complex mixtures - through the use of the FT-IR dedicated computer to do spectral arithmetic.

Thus, as separation techniques are applied, IR should be used to monitor the separation and identify the separated classes of species. Spectral subtraction should be used to magnify the differences between the separated fractions. Whenever possible, this spectral arithmetric will be used to minimize the need of chemical separations.

Used in this manner, IR will provide a major source of structural information to evaluate the HPLC separation and to determine whether or not more such separations are needed. When the IR data are combined with NMR data even more information can be

obtained. Combining IR and NMR with good separation techniques and subsequently with GC-MS and GC-IR, utilizes the most powerful tools available for organic compound identification.

(ii) Fourier Transform Infrared Spectroscopy

Fourier Transform infrared systems (30) differ from conventional dispersive infrared spectrophotometers in that conventional infrared spectroscopy uses a monochrometor to generate the spectral information whereas an interferometer is used for this purpose in Fourier Transform infrared spectroscopy (FT-IR). The use of an interferometer to generate spectral information in the form of an interferogram (light intensity versus time) necessitates a second difference between the two types of infrared spectroscopy. This difference is that FT-IR systems use a dedicated digital computer to obtain the Fourier Transform of the interferogram, converting it to a conventional infrared spectrum (light intensity versus wavelength or frequency). These two differences lead to the following two major advantages of FT-IR over conventional infrared spectroscopy:

- Using an interferometer results in a substantial gain in energy or light throughout as compared to a monochromotor. This gain in energy results from the fact that all wavelengths of light are examined simultaneously in an interferometer and no energy is lost (as in a dispersive instrument by examining the light one wavelength at a time). This additional energy can be used in one of several ways: (a) for faster scan speeds (as fast as 0.6 sec.), (b) for up to a 30-fold increase in signal-to-noise ratio, or (c) for 10^2-10^3 greater sensitivity.

- The availability of a dedicated computer offers several major data-handling advantages. Not only can spectra be ratioed against each other to remove absorption bands due to background materials, but the computer can be used to perform spectral arithmetic. Thus, spectra can be added or subtracted from each other and also multiplied or

divided. In this way, the spectra can be adjusted in size, and unwanted components can be removed from the spectra without the necessity of chemical separation. This ability to utilize a computer is not unique to Fourier Transform spectroscopy, i.e., in theory a computer could be attached to a conventional dispersive infrared spectrophotometer. However, in practice, this is rarely done, whereas whereas all Fourier Transform systems use a computer. Thus, from a practical standpoint, the use of a computer is a major advantage in FT-IR systems.

(iii) Experimental Details

Most experimental details (except for some instrumental parameters) are equally applicable to either Fourier Transform infrared systems or to conventional dispersive infrared spectrophotometers. Therefore except where noted, all of the following experimental sections apply to both types of infrared spectroscopy.

Method of Running Samples. Whenever possible (the vast majority of the time), the spectra should be obtained on a sample prepared as a film on an infrared transmitting crystal. In a few instances, the sample may be a highly light-scattering solid which is difficult to run as a film. In these cases the spectra should be obtained on a sample prepared by the pressed disk (KBr) technique. Details of both the film and pressed disk techniques can be found in Reference 31.

Preparation of Samples. As for NMR (See Section 4b, Preparation of Sample), the sample will come from HPLC fractionation as a dilute solution, which therefore will necessitate removal of the solvent. The same methods of solvent removal as given in Section 4b are applicable here with one small exception. For infrared studies it is only necessary to concentrate the solvent-sample system rather than to take the sample to

dryness. After the solution is concentrated to a few drops, the remainder of the solution is placed on the infrared crystal and the remaining solvent allowed to evaporate. If at this point, usuable infrared spectra can not be obtained, the sample should be removed from the infrared crystal and the pressed disk (alkali halide) technique used to prepare the sample.

Since some of the HPLC solvent systems contain water, it will be necessary to then use water resistant infrared crystals such as AgCl or Irtran. For all other solvents the common infrared crystals (NaCl, KBr) can be used.

If time is not a problem, it will be beneficial to use the solutions used for NMR rather than the HPLC solutions to prepare the infrared samples. The NMR samples will be either in the same solvent as used for HPLC or in a better solvent (for infrared purposes of evaporation) than the solvent used for HPLC. In addition the NMR solution will require less concentration than the HPLC solution.

Micro Sampling Techniques. During the separation procedures most of the samples will be of reasonable size and conventional infrared sampling techniques (as described above) can be used to prepare the sample for the screening process. However, as the separations proceed, the separated fractions can approach a size where micro sampling techniques will be needed. It is much more difficult to select a standard technique for micro samples than for conventional-sized samples. Not only does the handling of micro samples and micro sampling equipment require experience, but there are large differences in the micro techniques used by various laboratories. Therefore, while some micro techniques will be

suggested, experience in handling micro samples is so important that each individual will probably get the best results from the technique most familiar to him.

For practically all solids and many liquids, the micro pressed disk technique is recommended. It is the most universal technique and generally greater sensitivity is achieved than with a micro film technique. This micro-pressed disk technique requires the use of a beam condenser, micro-disk holders, and a press for producing the micro-pressed disks. All of these are commercially available.

For liquids where the micro-pressed disk technique is not applicable, a micro film technique can be employed. Here the film is constrained to an area on the infrared crystal which is the size of the infrared beam at the focal point of the beam condenser. This can easily be done by shaping the infrared crystal to this size or by digging a groove of this size in the infrared crystal.

It should be emphasized here that one of the main reasons for recommending FT-IR as opposed to dispersive IR spectroscopy is the greater sensitivity of FT-IR systems. In general a microgram of sample is needed to obtain a reasonable dispersive infrared spectrum, while only 10-100 nanograms is needed for an equivalent spectrum using an FT-IR system.

Instrumental Parameters. While there is basically only one mid-infrared Fourier Transform system available commercially, there are numerous commercially available dispersive infrared spectrophotometers. Thus it can be difficult to define meaningful instrument parameters since even the parameter nomenclature can vary from instrument to

instrument. However, some general remarks can be made and the details of good spectrophotometer conditions and operation can be obtained from Reference 31.

First it is necessary to consider whether or not all of the available instruments will give satisfactory spectra. Certainly all Fourier Transform infrared systems will yield high performance spectra. FT-IR systems differ from dispersive spectrophotometers in that interferometers give constant resolution over the entire wavelength range. In addition, FT-IR systems have greater wavelength accuracy than dispersive spectrophotometers. For these reasons, coupled with the FT-IR advantages previously listed, it is strongly recommended that FT-IR systems be used for all IR studies.

However, at the present time there are only approximately 75 FT-IR systems in the world as compared to many thousands of dispersive infrared spectrophotometers. Thus not all laboratories have access to FT-IR systems and some of the screening work undoubtedly will utilize spectrophotometers rather than FT-IR systems. While this may be necessary, it must be remembered that using a dispersive spectrophotometer will result in a definite sacrifice of sample information.

When using a dispersive infrared spectrophotometer care must be exercised in choosing an instrument which is capable of achieving the necessary performance specifications. In general most of the instruments costing less than $6,000 do not meet the necessary requirements because they lack the flexibility needed for the varied screening samples. Obviously, the spectrophotometers costing over $20,000 give the highest quality spectra (of the dispersive instruments) and their use is recommended when FT-IR systems cannot be used.

In general the instrument parameters used for spectrophotometers should be those given in Reference 31, while good operating conditions for FT-IR systems are listed in Reference 30, FT-IR spectra should be run at a resolution of 4 cm^{-1} (boxcar apodization), while dispersive IR spectra should be obtained at a slit width which yields an average resolution of 4 cm^{-1} over the spectral range (at least 3800-600 cm^{-1}). Sample thickness should be adjusted so that the strongest absorption band gives about 10% transmission. In many cases several sample thicknesses (both thicker and thinner) will be desirable (especially in FT-IR where spectra are to be subtracted). Noise levels should not exceed 2% peak to peak. The infrared instruments should be purged by dry gas or evacuated so that atmosphere absorption should not exceed the allowable noise level.

It is certainly desired that the wavelength accuracy approach $\pm$10 cm^{-1} above 2000 cm^{-1} and be less than that below 2000 cm^{-1}. The wavelength readibility should be better (for sharp peaks) than 10 cm^{-1} at wavenumbers greater than 2000 cm^{-1} and better than 5 cm^{-1} below 2000 cm^{-1}. To do this it is necessary to use charts greater than 8-1/2 by 11 inches.

4. (d) High Resolution Mass Spectrometry (HRMS)

High resolution mass spectrometry is periodically used as a screening technique for organic effluent samples, but in this instance the "Screening" is of a rather different nature to that generally intended in this manual. HRMS screening is most useful in searching for specific trace components, but is not readily able to give useful data regarding the general nature of organic species present in a complex mixture. For example, if the objective of an industrial effluent analysis is to determine whether any of several predetermined hazardous materials are present in the emission, then HRMS screening will provide very useful data, and will readily tell the analyst whether the selected compounds are absent in the sample, subject to the recognized detection limits of HRMS techniques. This type of HRMS screening makes use of peak matching (32) to determine exact fragment masses, and thus if fragment ions of the correct empirical formulae for fragment ions of a sought compound are absent, then the sought compound is judged to be absent. However, if fragments of the correct empirical formulae for the sought compound are present, this does not necessarily mean that the sought compound itself is present, since the apparently correct fragments could possibly have arise through the presence of an isomer or analogue of the sought compound. Thus, this type of HRMS screening will indicate whether a particular compound is absent, but is unable to definitely indicate that a compound is present. Screening studies of this type might more properly be termed "negative screening".

We would not anticipate that negative screening by HRMS would be a very widely applied technique, since many analytical studies are predominantly concerned with determining what species are present, which

precludes negative screening. Nevertheless, occasions will arise when the appropriate Government agency will require to know whether a specific effluent is being emitted from an industrial facility, and in such instances HRMS negative screening will be the screening procedure of choice to determine whether the specific effluent is absent or not, before resorting to other powerful analytical techniques, such as GC-MS and GC-IR, in order to ascertain whether the presence of the compound can be confirmed, and then to carry out quantification if necessary.

Because suitable standards for general chemical ionization HRMS are not available at the present time, HRMS is limited to electron impact ionization in most instances. The detection limits of HRMS is thus strongly dependent upon the ease of ionization of the compound being sought. For a relatively polar compound with large charge separations and ease of fragmentation, a reasonable detection limit may be well over a hundred nanograms, possibly a few hundred nanograms. For compounds that fragment with difficulty, such as highly aromatic species, and especially POM, the detection limit will be appreciably lower and might typically fall in the 10 to 30 nanogram range. It is possible to optimize sensitivity for HRMS negative screening by reducing the ionizing potential in order to reduce the initial excess energy of the molecular ion. However, this process must not be taken too far, when the ionization efficiency of the species sought would begin to fall rapidly.

Sample should be introduced into the HRMS by probe insertion; a portion of the organic extract will first be carefully evaporated to remove excess solvent in the glass micro-vial which is situated at the tip of the probe. The sample probe may be maintained at a constant temperature, but

is generally more useful to use a thermally programmed probe, which will provide some degree of separation of the sample, which can make interpretation of the data a little simpler. Several recent studies have described the utility of thermally programmed sample inlet probes (33).

In summary, it is expected that HRMS negative screening will find limited usefulness, except in instances where only specific compounds are being sought. Even in this case, the generally rather high detection limits of HRMS makes the technique somewhat unattractive as an individual screening technique.

4. (e) Evaluation of Screening Data

Determination of the complexity of a given HPLC fraction by the above-mentioned spectroscopic screening techniques leads to a decision-making point regarding whether the next step should be further separation, identification and quantification, or discard the sample due to lack of compound presence.

If the sample is of sufficient complexity or of mixed classes, further HPLC separations, as discussed in Chapter 5, must be performed prior to identification and quantification. Such a step would result in a recycling of the samples through HPLC separation and spectroscopic screening techniques until one class, a simple mixture of classes or a single component, suitable for identification and quantification, can be isolated.

If the screening procedures show the presence of only one class of compounds, a very simple mixture, or a single component, specific identification and quantification by one of the techniques discussed in

Chapter 6 can be performed immediately.

In the case of a simple effluent sample mixture, several of the HPLC class separation samples may not contain any compounds. If spectroscopic screening procedures show a blank sample, then the fraction should be thus noted and the sample discarded without further analysis.

5. ADDITIONAL SEPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In chapter 3, we discussed the power of sequential analysis using HPLC to affect a separation of a complex mixture. Frequently, this may prove entirely successful for compounds of interest. In dealing with complex multicomponent fractions however, further separation is often essential before identification and quantitation can be achieved. Fortunately, there are additional modes of HPLC available to the analyst in his quest for total analysis. In this chapter, these additional chromatographic modes will be discussed, their selection will depend on the results of screening and prior separation and therefore a coordinated analytical effort is a necessity.

(a) Bonded Phase Chromatography

We have used bonded phase chromatography in step two of sequential analysis to obtain a initial class separation. The same reverse phase column can be used either isocratically or with gradient elution, to further separate the sample. Optimum solvent conditions can be estimated from the class separation already performed. For example, if a fraction containing multiple peaks was collected when the composition of the mobile phase was between 20 and 25% methanol, that fraction can be chromatographed

isocratically at 20% methanol or a gradient performed from 15 to 30% methanol. This second separation will vastly improve the resolution of the mixture. Since the column and solvent consitions are available, this is probably a good first step in additional separation.

Normal bonded phase chromatography in which the stationary phase is polar (alkyl amino or cyano groups are bonded to the surface) can also be used as an additional separation mode. Different selectivities can be obtained using these phases since the interactions of various functional groups with the surface is markedly different. When employing these phases, usually non polar eluents are used; gradients can be run using heptane and isopropanol, for instance, as solvent extremes.

When very polar or ionic compounds have been diagnosed by screening, reverse phase bonded chromatography or ion exchange (see later) is the separation mode of choice.

Selectivity can be largely controlled by the pH of the mobile phase when ionic compounds are chromatographed on a reverse phase column, and therefore difficult separations can be achieved. Very acidic or basic compounds are best separated by ion exchange.

(b) Liquid Solid Chromatography

Liquid solid, or adsorption, chromatography is the oldest and most widely used separation mode. In adsorption chromatography, an adsorbant, such as silica gel or alumina, is used as a polar stationary phase, and non polar to polar mobile phases are used to elute components. One of the big advantages in using this mode is that a wealth of information can be obtained from thin layer chromatographic literature that can be applied to HPLC separations. Only slight changes in conditions are necessary

to transfer TLC data directly to a column separation.

Another fact which has contributed to the wide use of silica gel as an HPLC stationary phase is that the first high performance microparticle columns which were successfully slurry packed were of silica gel.

Liquid solid chromatography is best used for non-ionic compounds that are not amenable to G C. Compounds of different chemical type or differing numbers of functional groups are easily separable by adsorption. The best resolution of chromatographic peaks are achieved on adsorption columns, so if total separation is necessary, LSC should be tried, LSC has difficulty in separating homologous series or compounds differing in the extent of aliphatic substitution. Very polar or ionic compounds are also difficult to elute using adsorption and are best done by reverse phase or ion exchange chromatography.

(c) Ion Exchange Chromatography

Ion exchange chromatography has always been the established method of separating ionic species. It has therefore been used extensively in biochemical separations, but its usefulness in treating general complex samples is limited. However, after sequential analysis has been performed and an ionic fraction isolated, ion exchange chromatography can be used to affect a separation.

Ion exchange chromatography is carried out with stationary phases which contain charge-bearing functional groups. The mechanism is frequently simple ion exchange as follows:

$$X^- + R^+Y^- \rightleftharpoons Y^- + R^+X^- \quad \text{(anion exchange)}$$

$$X^+ + R^-Y^+ \rightleftharpoons Y^+ + R^-X^+ \quad \text{(cation exchange)}$$

where X = sample ion, Y = mobile phase ion and R = ionic sites on the exchanger. The solute ion competes with the mobile phase ion for the ionic sites on the stationary phase. For this reason changes in the pH or ionic strength of the eluent have a dramatic effect on retention for further discussions concerning retention on cationic or amonic exchange support see Reference 12.

Both reverse phase liquid chromatography and ion exchange can be used to separate ionic compounds. With proper control of pH, reverse phase chromatography is capable of many separations of weak to moderally ionic compounds. Compounds which are strongly ionic can be chromatographed by ion exchange.

(d) Evaluation of Additional Separations

Evaluation of the additional separations will be carried out by the spectroscopic procedures described in Chapter 4. The objective of this evaluation is fully described in Chapter 4, and is to determine what classes of organic compounds are present in the further separated fractions in order to facilitate qualitative and quantitative analysis described in Chapter 6.

6. IDENTIFICATION AND QUANTIFICATION

(a) Gas Chromatography (GC), and High Performance Liquid Chromatography (HPLC)

Introduction

Gas chromatography is a powerful tool for separations of complex organic mixtures. In combination with selective detectors, particularly

mass spectrometry, it is among the most powerful instruments available to the analyst. The principles of HPLC have been extensively discussed in Chapter 3, and thus only limited additional comments on its role in quantitative analysis will be made in this chapter.

There are a number of gas chromatographs commercially available which are suitable for complex organic mixtures. It is important that the column oven is programmable at various rates up to about 400 C. The facility for dual columns and dual detectors is sometimes useful, especially when column effluent is split between two detectors such as a general purpose flame ionization detector (fid) and specific detectors such as a flame photometric detector (fpd) or electron capture detector (ecd).

The most important part of a gas chromatograph is the chromatographic column. There are various types of column ranging from packed, through support coated open tubular (SCOT), to high resolution capillary columns. Column packings include silica gel, alumina and polymers such as the Poropak and Chromosorb series, but the coated diatomaceous earth type are very commonly used. There are a great number of column packings commercially available; many of these have been developed in response to specific problems, whereas others have a more general usefulness. The listing given below in Table 5 does not pretend to be a complete inventory of column packings and coatings, but is presented to serve as a preliminary guide. Useful data may be frequently found in chromatographic supply manufacturers catalogues, in addition.

TABLE 5. ORGANIC SPECIES SEPARABLE BY VARIOUS GC COLUMN TYPES

Compound Type	Column Type
Acids C_1-C_9	Chromosorb 101
C_1-C_{18}	FFAP
Alcohols C_1-C_5	Poropak Q, Chromosorb 101
C_1-C_{18}	Silar 5CP, Carbowax 20M, FFAP
Polyalcohols	FFAP
Aldehydes C_1-C_5	Poropak N, DC-550, Ethofat
C_5-C_{18}	Carbowax 20M, Silar 5CP
Amines	Poropak Q/PEI, Poropak R
	Chromosorb 103, Pennwalt 223
Amides	Versamid 900, Igepal CO-630
Esters	Poropak Q, Dinonylphthalate
	Chromosorb 101 or 102
Ethers	Carbowax 20M, Silar 5CP
Freons	Poropak Q, Chromosorb 102
Glycols	Chromosorb 107
Halides	OV-210, FFAP
Hydrocarbons C_5-C_{10}	OV-101, SE-30
Aromatic	Silar 5CP, Carbowax 20M
Olefins C_6-	DC-550, DC-703
POM	Dexsil 300, OV-101, SE-30
Ketones	Poropak Q, Chromosorb 102, FFAP
Pesticides	OV-101, OV-225, OV-1, OV-17, SE-30
Phenols	OV-17, Silar 5CP, Carbowax 20M

A significant proportion of GC separations may frequently be achieved using conventional packed columns. Capillary columns are becoming more widespread but their use requires greater skill than packed columns. It is anticipated that the use of capillary columns in the analytical strategy presented here will be restricted to the separation of isomers and other groups of similar compounds, since many of the difficult separations will already have been accomplished by means of quantitative high pressure liquid chromatography, which has been described earlier.

Gas chromatography can frequently provide good resolution of complex mixtures of organic compounds, and for this reason it is commonly used in organic analysis. An obvious limitation of the technique is that involatile materials are not amenable to this method of analysis. Involatile materials are most expediently analyzed by liquid chromatography, as described earlier.

A typical organic extract should normally be concentrated to a few hundred microliters, before subjecting one or two microliters to GC analysis. When an extract has been separated by high pressure liquid chromatography, it is usual to concentrate this extract to about fifty to a hundred microliters prior to GC analysis. The choice of solvent for GC is not generally critical; commonly used solvents such as methylene chloride, acetone, or benzene generally elute significantly before any of the compounds of interest. If interference between solvent and sample peaks is observed it may be necessary to change solvents or GC columns.

The sensitivity of GC with conventional fid depends to a large extent upon peak sharpness and the level of noise or other interfering peaks. In the absence of appreciable interference, a sensitivity of 50 ng/μl

may reasonably be obtained for hydrocarbons using fid. With fpd or ecd, for sulfur compounds or chlorinated pesticides for example, a sensitivity or between one and two orders of magnitude better than this is reasonably obtainable in most instances.

Screening Studies with GC

While GC is generally most useful for quantification, it is nevertheless possible to obtain some useful preliminary screening data in addition. The use of GC with fid detection as a preliminary screening tool is somewhat limited by a lack of selectivity, nevertheless GC/fid screening can provide some useful data regarding the general nature of compounds which may be present in a sample. The most useful approach to GC screening is to use a generally applicable column such as 3% OV-17 or OV-101, and program at about 6 C min^{-1} from 100 to 300 C. See earlier GC column listing. Few commonly encountered organic compounds of moderate to zero polarity will not give reasonably good peaks under such conditions. However, all that GC/fid screening studies will be able to ascertain is whether or not organic compounds are likely to be present in a given sample, and whether they may be reasonably resolved by GC without use of additional HPLC separation. Rather more information can be gained from GC screening by the use of selective detectors such as fpd or ecd, but the use of such selective detectors rather presupposes that only specific information is required from the sample. For example, if we only wish to know whether a sample contains sulfur compounds or not, the obvious way to approach this problem is by use of an fpd with a sulfur filter. It is a useful practice when doing screening of this nature to obtain a simultaneous fpd and fid

output which will permit the specific fpd peaks to be related back to the fid or mass spectrometric ion current trace (through the fid trace).

The nature of the GC column can give only limited information regarding the nature of compounds present, and it is not recommended that an appreciable effort should be devoted to such a study. For example, compounds eluting only at relatively high temperatures on a relatively non-polar column such as OV-17 or OV-101, are probably highly polar materials such as dialdehydes or other nonfunctional compounds, or large, bulky compounds such as POM. Compounds which are only resolved with difficulty on relatively polar columns such as Silar 5CP or Carbowax 20M are likely to be relatively small molecules of moderate polarity.

Perhaps the most valuable data obtained from GC screening, however, is simply to determine whether the HPLC separations previously carried out have provided a sample in which the individual compounds are sufficiently well resolved for further qualitative and quantitative analysis on the most appropriate GC column. If this is not found to be the case, additional HPLC separations must be carried out before further analysis can be attempted.

Quantification with GC and HPLC

Gas chromatography is probably the most commonly used tool for quantification in organic analysis. High performance liquid chromatography has recently been used more extensively for quantification on account of significant improvements in instrumentation. In order to carry out quantitative analysis by either GC or HPLC, it is necessary that the compounds of interest should be reasonably well resolved from other peaks in the chromatogram. Furthermore, it is highly desirable that pure samples of the compounds

sought should be available; if the components sought are not available, an approximation may be made through use of their analogues. An internal standard, or standards, must be chosen which will elute reasonably close to the peak(s) of interest, and will itself be free from interference from other chromatographic peaks.

Calibration is carried out by preparing a known mixture of internal standard(s) and compounds of interest, and obtaining a chromatogram for several different amounts of the mixture. The response factor for each peak may be determined by measuring height, area, or preferably by use of an automatic integrator or computer integration routine. Sufficient calibration chromatograms are run until a consistent response ratio between the internal standard(s) and the chromatographic peaks of interest is obtained. When response ratios between every compound of interest and an internal standard have been satisfactorily determined, the appropriate internal standards are added to the unknown mixture in amounts which are estimated to be of the same order of magnitude as the peaks which are being determined. GC analysis of the complex mixture plus internal standards is now carried out using the same chromatographic conditions at which the response ratios were originally obtained; from the response factors for the compounds sought in the complex mixture, and for the known amounts of internal standards together with the previously determined response ratios, the absolute quantity of each of the compounds sought in the complex mixture may be readily determined.

When it is possible to use specific detectors, such as GC-ecd for halogenated pesticides, GC-fpd (sulfur specific filter) for sulfur compounds, or HPLC-fluorescence for POM compounds, it is often possible to carry out

quantification without obtaining good peak resolution on the chromatogram. This may be feasible because the sensitivity of the specific detector is very low for all but the compounds of interest, which permits previously interfering peaks to be neglected on the specific detector chromatogram.

The internal standards chosen for GC and HPLC quantification are usually similar in nature to the compounds which they are being used to determine, although this is not always mandatory. For example when carrying out GC quantification of POM species in combustion emissions an uncommon alkylated or phenylated POM compound is often chosen as an internal standard; it is of course important that the standard chosen does not occur in the complex mixture under analysis. In the GC analysis of C_{12} to C_{26} hydrocarbons from an oil-spill sample, it is not uncommon to add an absent C_{32} hydrocarbon to serve an internal standard for the mixture. When the highest accuracy is not sought, it is an accepted practice to assume that the chromatographic response for each member of a class of compounds is the same. Thus the POM compound 9-phenylanthracene could be used as an internal standard for all other POM compounds, each being assumed to have the same response as the internal standard, and the response ratio of each compound to the internal standard equal to unity.

The accuracy and reproducibility of GC and HPLC quantification using internal standards is generally better than $\pm$10%. The reproducibility may be readily determined while obtaining the response ratio calibration for each compound sought.

6. b) Gas Chromatographic-Mass Spectrometric Analysis (GC-MS)

Gas chromatographic-mass spectrometry is presently a widely used and powerful tool for organic analysis. It has become common practice to interface a GC-MS system with a dedicated mini-computer, and a variety of output units such as a CRT teletype, or XY plotter, and a high-speed line printer, it is probably true to say that the GC-MS analysis contemplated in this manual could not be accomplished without an interfaced mini-computer.

Electron Impact and Chemical Ionization

Mass Spectra may be obtained by electron impact ionization (EI) or by chemical ionization (CI); in the latter mode, sample ionization is accomplished by means of an ionized reagent gas. In CI GC-MS analysis, the reagent gas, such as methane or isobutane, is most commonly introduced through its use as the GC carrier gas, no molecular separator being used at the interface between the GC and MS. The MS source pressure for CI with methane, for example, is typically as high as 600 microns, and thus the far higher concentration of methane than sample in the source ensures that sample ionization will occur exclusively by collision with ionized methane. Chemical ionization results in a rather different mass spectrum to electron impact in view of its being a much lower energy process; CI spectra are characterized by less extensive fragmentation of the molecular ion, and the fragmentation which occurs generally proceeds through loss of neutral molecules and appreciably more stable fragments than is the case with EI (34). It is usual to observe a protonated molecular ion in CI,

and this is frequently accompanied by two adduct ions at M+29 and M+41, in the case of methane CI, caused by the addition of $\dot{C}_2H_5$ and $\dot{C}_3H_5$ radicals in the source of the mass spectrometer. Such adduct ions are generally diagnostic for the protonated molecular ion, and thus it is frequently possible to quickly assign a molecular weight during CI GC-MS analysis.

Since CI analysis is still relatively new, EI analysis maintains a significant advantage which is well suited to the analysis of complex organic mixtures. Over the past few years extensive data files of EI spectra have been built up, many of these giving particular emphasis to toxic and hazardous substances. As yet, no comparable files exist for CI spectra. It is not unusual to possess a data file of 25,000 or more EI spectra with which to carry out spectral matching as an aid to identifying unknown compounds. Spectral matching is often carried out by gaining access to spectral files in a central computer, several laboratories sharing the same data file. In our experience, it is considerably more convenient to store a spectral matching data file on disk, and by means of a dual disk drive assembly interfaced with the GC-MS minicomputer it is then possible to carry out instantaneous spectral matching in the GC-MS laboratory. In any case, spectral matching is a very useful aid to the interpretation of unknown EI mass spectra, although it will often fail to suggest an immediately satisfactory spectral fit. Spectral matching often provides a useful guide which when coupled with other analytical data, such as a molecular weight from a CI mass spectrum, liquid chromatography separation data, and NMR and IR screening studies; correlation of such combined data can frequently lead to a reasonable interpretation of the mass spectrum.

Correlation with Separation and Screening Data

GC-MS analysis does of course presuppose that the complex mixture subject to analysis is capable of separation by gas chromatography and an obvious limitation is compounds which are too polar to be satisfactorily separated by GC. Such compounds may be routinely analyzed by HPLC, as discussed earlier. A great deal of valuable data on an unknown sample will be obtained by screening studies even before GC-MS analysis is attempted, and the complexity of the mixture will have been significantly reduced by the HPLC separation scheme which has been discussed previously.

HPLC will initially be used to separate a complex mixture into three to five fractions by GPC on the basis of molecular weight, and then each of these size fractions will be separated into as many fractions as appears desirable by silica gel or reverse phase HPLC in order to provide relatively simple mixtures for the subsequent stages of analysis. Following the complete HPLC separations, probably into about 20 fractions, screening studies using IR, NMR, HRMS, and GC will have been carried out as appears appropriate.

Each fraction of the complex mixture of a given molecular weight range will be separated by HPLC on the basis of polarity into a number of well resolved fractions. The very non polar fractions will consist of aliphatic hydrocarbons and will not be subject to GC-MS analysis, since these species are more efficiently analyzed by GC alone. The most polar fractions from the HPLC separation scheme will be unsuitable for GC separation on account of their high polarity and involatility, and will

be characterized by further HPLC separation together with IR, NMR, and low resolution MS as required. However, the HPLC fractions in between these two extremes will range from non polar POM species to polar polyfunctional compounds, and these will be identified by GC-MS (and possibly GC-IR) preceeded by extensive screening of each HPLC fraction by the other analytical techniques as discussed earlier.

Whether CI or EI mass spectra, or both, are obtained will depend to a large extent on the information available from the preliminary screening and HPLC separation data. For fractions containing the POM species, there is little point in obtaining both EI and CI spectra, since fragmentation is almost negligible and uninformative in both cases. However, the preliminary screening data will make it clear that POM species are present, and CI mass spectra will provide molecular weights which when coupled with chromatographic data will permit unambiguous compound identification.

For HPLC fractions containing monofunctional compounds, such as aldehydes and phenols, CI mass spectra may frequently provide sufficient confirmatory data, especially when coupled with the information from the screening analysis. For example, if an HPLC fraction is known from screening to contain primarily aromatic aldehydes, it should be relatively straightforward to assign benzaldehyde, tolualdehydes, and higher alkyl benzaldehydes from the CI spectra, since the only prominent fragmentation will be loss of 28 mass units for CO, to leave the carbon skelton. The mass spectra are unlikely to give guidance regarding isomers, but this information may be inferred from chromatographic data, or possibly GC-IR data.

For HPLC fractions which contain polyfunctional compounds, or a mixture of compounds of different functionality, the most expedient approach in GC-MS analysis would be to initially run EI spectra, and initially rely upon spectral matching, and fragmentation interpretation, to suggest some possible structures for the compounds present. The available screening data will greatly assist in narrowing the choice from the list of spectral matches. It may not at this stage be possible to make a good assignment of some of the compounds present, and it would probably be most helpful to additionally obtain CI mass spectra. The CI spectra would further narrow the possibilities by providing a probable molecular weight for the compounds of interest, and the CI fragmentation may give further valuable data necessary to arrive at a structural assignment. CI fragmentation for moderately polar species can be rather more informative than for less polar compounds, since the greater charge separation in the molecule may be more likely to promote fragmentation; for the same reason, EI fragmentation of very polar species may often be too extreme to be of much value.

GC-MS Quantification, and Other Useful Computer Routines

The basic routines available with all commercial mini-computers for GC-MS are 'RGC' (reconstructed gas chromatogram) and a mass spectrum printing routine. It is highly desirable that a CRT teletype unit should be available for instantaneous display and manipulation of data; we will assume in this discussion that a CRT unit is available, although the routines are almost identical when used with a more time consuming XY plotter.

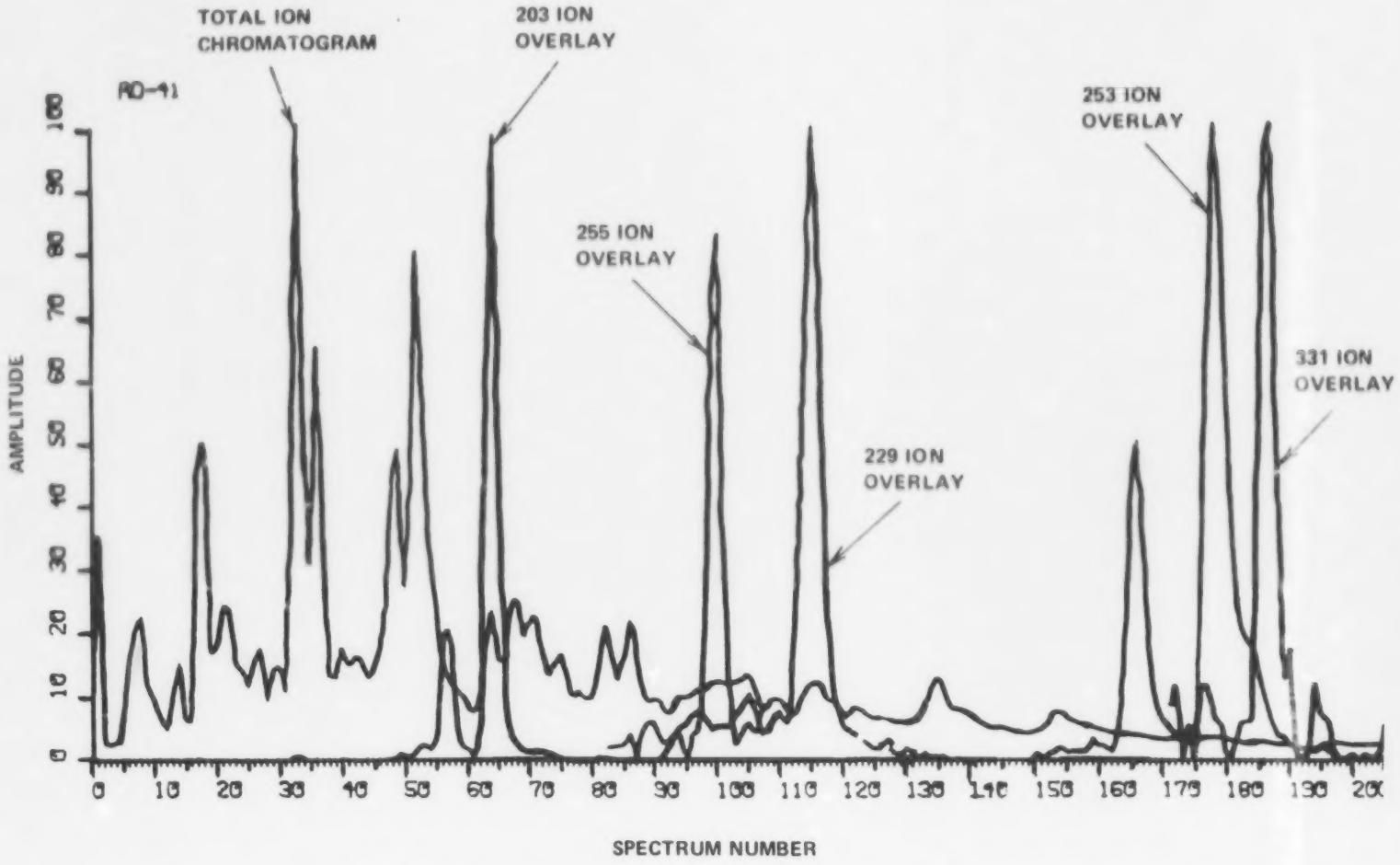


FIGURE 9. RECONSTRUCTED ION CHROMATOGRAMS OF RESIDUAL OIL COMBUSTION EFFLUENTS

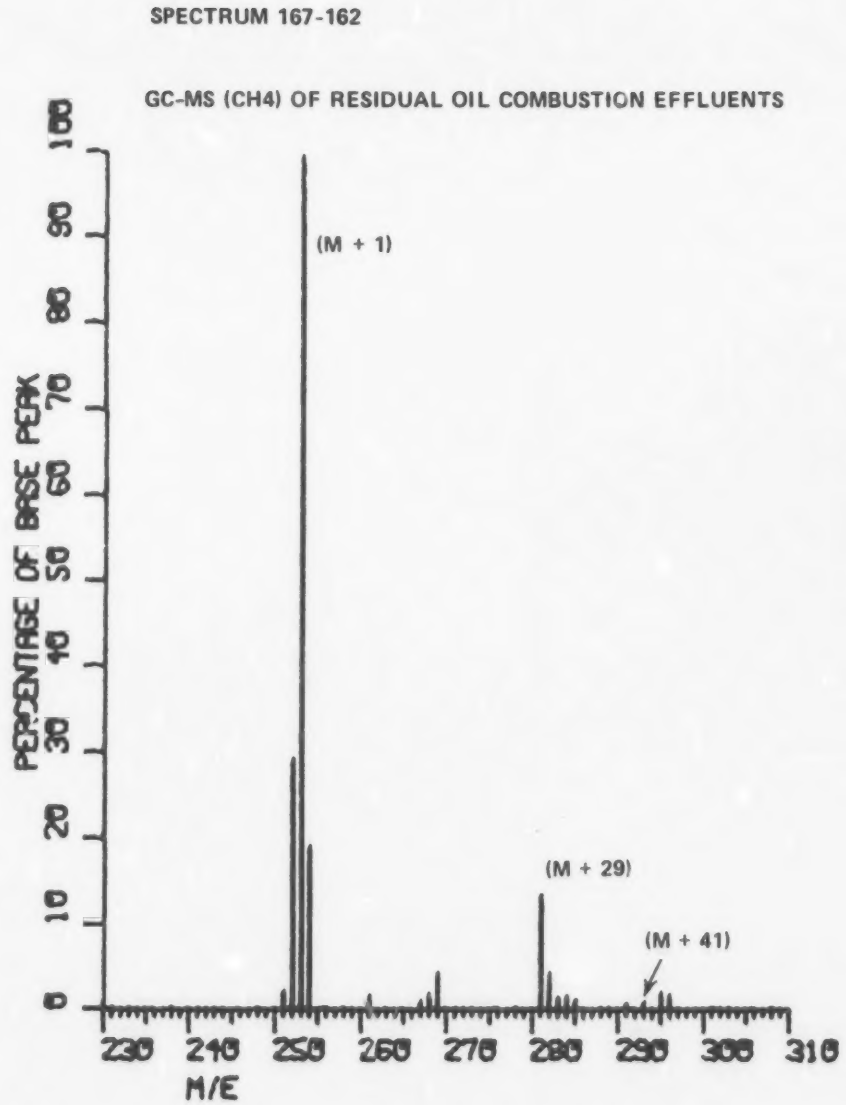


FIGURE 10. MASS SPECTRUM OF BENZFLUORANTHENES

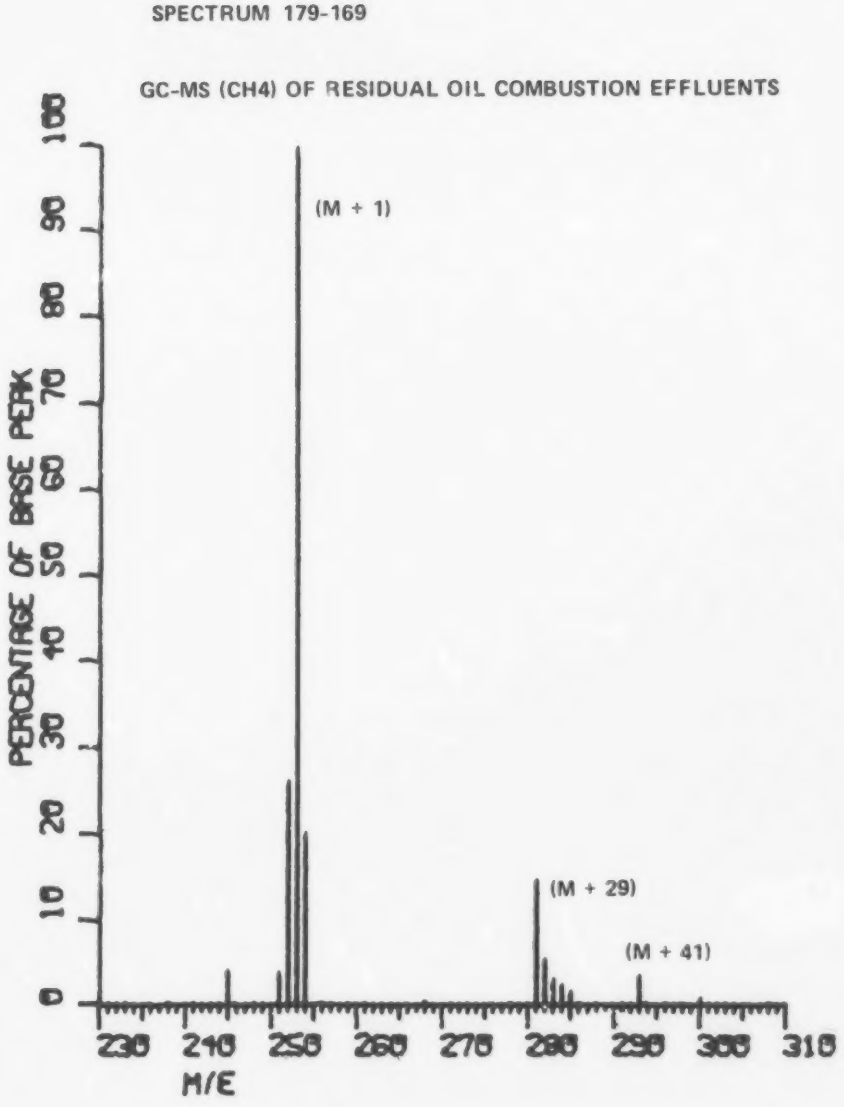


FIGURE 11. MASS SPECTRUM OF BENZPYRENES

Modifications of the RGC routine are invaluable for the location of minor chromatographic peaks, and also for performing rapid quantitative analyses. The normal RGC plot consists of a reconstructed chromatogram which contains ions of all mass numbers, see for example the 'total ion chromatogram' in Figure 9. In order to locate the GC peak for a compound whose mass spectrum is known, RGC plots containing prominent ions in the mass spectrum of this compound may be made. These RGC's should be overlaid upon the original total ion RGC, maxima in the RGC specific ion overlays will occur at the spectrum number corresponding to the compound of interest in the total ion RGC.

For example, Figure 9 shows a portion of the GC-MS analysis of residual oil combustion effluents. In this case, the sample extract was subjected to liquid chromatography on silica gel to isolate the hydrocarbon and POM species. Several individual ion overlays are shown superimposed upon the total ion chromatogram. The 253 ion overlay shows maxima at spectrum numbers 166 and 179, although it is apparent that no peaks are visible on the original total ion chromatogram. If the spectra at spectrum numbers 166 and 179 are displayed or printed out, it is evident that these two peaks are benzfluoranthene or benzpyrene isomers; the spectra are both characterized by a base peak at m/e = 253 (M+1) and adduct ions (M+29, M+41) at m/e = 281 and m/e = 293 as shown in Figures 10 and 11. In practice, spectrum 167 minus spectrum 162, and spectrum 179 minus spectrum 169 would be displayed in order to subtract any spurious background ion peaks due to column bleed, and the tailing from other chromatographic peaks. It is important that the presence of the

compound sought should be confirmed by printing out its mass spectrum in this manner, since spurious 'hits' sometime occur due to interfering fragments from other compounds.

Specific ion current integration is the basis for a very rapid quantification routine (3). Some mass spectroscopists use the total ion current for quantification purposes, and while this procedure may work well for well resolved peaks at high concentrations, the procedure unfortunately neglects the high specificity and sensitivity which the mass spectrometer is capable of providing. In order to obtain the specific ion currents due to minor peaks which are confused and overlaid by other major peaks, the precise position of the minor peak is firstly determined by means of the RGC overlay technique. For example, in Figure 9, the location of pyrene (spectrum 64) and fluoranthene (spectrum 56) is established by overlaying the 203 ion on the total ion chromatogram; 203 is the mass number for the protonated molecular ion of both these compounds. Peak width limits are then read off from the chromatogram, or located with a CRT cursor, and in this case are seen to be spectra 54 to 61 and spectra 61 to 69 for fluoranthene and pyrene respectively. Having established the peaks limits, another computer routine is used to sum the ion currents due to all of the prominent ions in the mass spectrum of the compounds of interest. The ion integration procedure is then repeated for an internal standard which was previously added in a precisely known quantity to the complex mixture. In this example, the internal standard used was 9-phenylanthracene, whose position is indicated by the 255 ion overlay in Figure 9, as spectrum 100. Quantification of the compound of interest is then achieved by ratioing the ion current of

of the compound of interest to that of the internal standard, and applying a previously determined calibration factor which allows for the difference in ionization efficiencies of the compound sought and the internal standard. Interference by fragmentation from other unwanted compounds can almost invariably be avoided by using CI (which minimizes fragmentation), and by careful choice of the fragment ions used for quantification. This quantification procedure has repeatedly been demonstrated to have an accuracy and reproducibility of better than ±15%; with care this figure may readily be reduced to below ±10%. The above quantification procedure has similarities to the widely used specific ion monitoring technique, which of course is not suitable for the analysis of large numbers of compounds in environmental samples on account of unavailability of isotopically labelled reference materials.

The choice of internal standards for GC-MS quantification should be made with greater care than for GC internal standards, since not only do we require a compound that elutes conveniently near to the compounds to be measured, and is not itself present in the sample, but we also require a compound which will give simple fragmentation in order to obviate interference from fragment ions from other materials during ion current integration.

The sensitivity for GC-MS quantification, and for GC-MS analysis in general, will always depend upon the nature of compounds being studied. For a compound such as an aliphatic dialdehyde with extensive fragmentation, and whose mass spectrum is necessarily weak, a sensitivity of 100 ng/µl or poorer would not be surprising. For compounds showing little or no fragmentation, such as POM species, a sensitivity of 1 to 10

ng/μl can routinely be obtained, sensitivities of an order of magnitude or more higher than this may be obtained if mass spectrometer conditions are optimized.

One of the most useful analytical routines is spectral matching, as previously mentioned. We consider that it is preferable to use a dual disk drive whereby the bank of reference spectra are stored on a separate disk which is readily accessible during mass spectral analysis. Ideally, the RGC of a fraction from HPLC separation should be displayed on the CRT, and a queue of mass spectra corresponding to RGC peaks should be stored in the computer memory by command from the teletype. When the queue is established, the matching routine may be activated and a chosen number of compound matches (with correlation coefficients) for each RGC peak may be printed by a high-speed line printer.

6. c) Gas Chromatographic-Infrared Spectroscopic Analysis

While infrared spectroscopy (IR) is the most widely used analytical tool for the identification of organic compounds, it has given way to mass spectrometry (MS) for the identification of organic compounds in vaporizable, complex mixtures. The reason for this was that the characteristics of mass spectrometry were well suited to coupling with a gas chromatograph (GC) so that GC could be used to separate the mixture and MS used to identify the separated compounds. The sensitivity and speed of MS was such that this could be done "on-line" or "on-the-fly". GC-IR studies used to require trapping and collection of each GC peak, and not only was this time consuming, but often there was not enough material available for this purpose.

With the advent of Fourier Transform infrared systems (FT-IR), "on-line" GC-IR became a reality. The extra energy throughput of the interferometer of the FT-IR system could be used to give complete infrared scans in as little as 0.5 sec. Thus scanning speed was certainly fast enough for "on-line" GC-IR without the necessity for trapping and collection of the GC peaks. While the sensitivity level is not as low as desired, it is adequate for many samples. In addition designs are available for modifying the infrared light pipe used for GC-IR and this coupled with the use of a liquid nitrogen cooled infrared detector has been demonstrated to lower the sensitivity to 400 nanograms per GC peak. This sensitivity range makes it possible to obtain infrared spectra "on-the-fly" of most gas chromatographic peaks. Not only would such modifications be fairly easy to make, but a complete unit incorporating these changes should soon be commercially available.

Thus GC-IR joins GC-MS as a routinely used instrument for the analysis of complex mixtures and will be an invaluable analytical tool for identification of the components separated by gas chromatography. It is important to appreciate that GC-MS and GC-IR techniques frequently complement each other. Since the bases for GC-MS and GC-IR identifications are fundamentally different, analyses using both techniques will provide substantially more compound identification data than either technique used independently.

The same remarks concerning the use of separation techniques to prepare the sample for GC-MS (Section 6b) will apply to the samples for GC-IR and need not be repeated here. Since the GC-IR system is fully automated, the sample is merely injected into the gas chromatograph and

the FT-IR computer automatically scans, collects, and stores the interferogram for each GC peak. The operator then has to manually instruct the computer to plot out each spectrum. Identification is made by standard infrared procedures, that of matching the unknown spectrum with a reference spectrum from available reference libraries of up to 150,000 spectra. This can be aided by computer search systems. When an exact match (with a reference spectrum) can not be found, the functional group information (available from the IR spectrum) can often be coupled with the MS data to uniquely identify an unknown compound.

6. d) Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy will be used for quantification of effluent samples in only two cases: (1) a single component isolated by the HPLC separation scheme, or (2) the very polar or ionic compounds which cannot be quantified easily by the other available methods.

It is desirable that quantitative work should be carried out by FT-NMR, if possible, in view of the advantages of this technique discussed in Chapter 4. Quantification in NMR terms means either a relative ratio of the resonances present in the sample or addition of a known amount of a standard whose resonance will not interfere with those of the sample. For ^{13}C spectra, quantification also means running nuclear Overhauser enhancement (NOE) experiments to ascertain the true spectral intensities. In this document, quantification will be discussed in general terms applicable to either ^{1}H or ^{13}C spectra.

Relative Ratios

In the case of relative ratios, a sharp resonance peak such as a $-CH_3$ singlet is chosen as a known. The intensities of the integrals for the other resonances are then compared to the known . In this way the relative number of protons can be determined for a single peak as a chemical shift region (i.e., aromatic, olefinic, aliphatic). Sample preparation and experimental details for this procedure are those given in Chapter 4. The spectral integration is carried out using the computer program package provided with the instrument in the case of FT-NMR, or by linear summation of the integration trace with conventional NMR. The limit of accuracy for this method is $\pm$5% provided no exchangable protons or interfering groups are present in the spectrum. It must be again cautioned that this is a relative ratio; one which does not take into account such factors as relaxation times which can mask the true peak intensities especially in ^{13}C NMR

Absolute Quantification

In the case of absolute quantification, the spectrum of the HPLC fraction to be quantified is run according to the guidelines given in Chapter 4. Once the appearance of the spectrum and its suitability for quantification have been determined, the spectrum can be scrutinized for a blank area or window in which no sample resonances appear or are suspected, and a suitable internal standard chosen with a resonance in this area. A good compound for consideration as an internal standard would be relatively unreactive with the class of compounds in the sample and would contain a sharp singlet, or several singlets which are easily integrated.

Integration is carried out by the computer program of each instrument, in the case of FT-NMR, or by linear summation of the integration trace. Quantification to ± 5% can be accomplished by comparing the integral of peak of known concentration to those for the compound.

Specific Problems in the Analysis of Mixtures

The general approach to running NMR spectra as outlined in Chapter 4 will normally produce spectra which provide sufficient information for screening HPLC separation fractions, or identification of specific compounds in simple mixtures. Occasionally, however, the interpretation of the NMR spectrum of a mixture may be complicated by overlapping peaks or line-broadening. Depending on the particular problem and the nature of the sample components, several approaches can be used to simplify the spectrum.

In simple mixtures what appears to be the spin-spin coupling system of a single compound, may in actuality be peaks from two or more components. Spin decoupling techniques should be used to determine the integrity of the miltiplet. If the system collapses to a single resonance, the multiple peaks are part of one spin system.

The presence of paramagnetic materials in a sample can cause significant line broadening, and in some cases a low resolution spectrum. The effect is significantly increased if the particle is ferromagnetic. These interferences, from the original sample or the result of contamination during sample handling (i.e., steel spatulas), may be removed by using a permanent magnet and decanting the solution.

To resolve overlapping in compounds which have one or more Lewis base groups, especially hydroxy groups, carbonyls, and amines, a shift reagent may be used. Shift reagents are normally compounds containing a metal from the lanthanide series which complex with the Lewis base. The perturbation of the shift reagent on the proton magnetic resonance spectra of the ligands results in spectral simplification. For an excellent review of rare earth shift reagents, references 34-37 should be consulted.

Finally, as stated before, the use of FT-NMR with a dedicated computer provides the additional opportunity for special data handling, such as spectral arithmetic. Although not currently available on all commercial spectrometers, spectral arithmetic is a powerful tool for the similification and interpretation of spectra. For example, suspected components may be subtracted, both simplifying the resulting spectrum and confirming the component's presence.

7. ANALYTICAL COSTS

The following analytical cost estimates are presented in order to serve as a guide in planning emission measurement programs in a cost effective manner. The costs quoted for each operation (see Table 6) make the assumption that the analytical operation being carried out involves a complex mixture of organic compounds. In our experience, the individual costs given are reasonably representative of those experienced by the major research laboratories in this country.

TABLE 6. APPROXIMATE COSTS OF INDIVIDUAL ANALYTICAL STEPS

	$	
Extraction	50	(per emission)
GPC	110	(per extract)
HPLC	110	(per fraction)
IR Screening	60	(per fraction)
NMR Screening	100	(per fraction)
HRMS Screening	150	(per fraction)
GC Quantification	300	(per fraction)
HPLC-NMR Quantification	250	(per HPLC Peak)
HPLC-IR Quantification	125	(per HPLC Peak)
GC-MS Qualitative	600	(per EI or CI run)
GC-MS Quantitative	600	(per EI or CI run)
GC-IR qualitative	500	(per run)
LC Quantification	50	(per LC Peak)

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